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Formulation and Biological Activity of Antineoplastic Proteoglycans Derived from *Mycobacterium vaccae* in Chitosan Nanoparticles

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Abstract

Although heat-killed suspensions of *Mycobacterium vaccae* have been tested clinically against tuberculosis and cancer, from a pharmaceutical perspective it would be advantageous to utilize isolated active components rather than the heat-degraded bacterial materials. In our laboratory we have isolated from *M. vaccae* a number of high-molecular-weight proteoglycans with considerable immunological and antineoplastic activity.

The structure of one of these, PS4A, obtained by extraction with boiling water, seems to consist of a basic unit with a 20-kDa protein core to which are attached glucans and O-methylated 4-kDa polysaccharides. The molecular weight is (approx.) 50 kDa, but because of self-association, that of the recovered high-molecular-weight fraction is greater than 150 kDa. A similar, but even larger, molecule (PS4α, MW $\sim$20 MDa) is obtained by cold extraction with 8 M urea. Both are active in-vivo against an S-180 murine sarcoma model but have no activity in-vitro, suggesting an antitumour effect involving activated macrophages. For this reason gelatin nanoparticles are unsuitable as a vehicle but chitosan seemed to be a promising alternative. In this report we describe the production of stable 600–700-nm diameter nanoparticles of chitosan without organic solvents. Adsorption and release of bovine serum albumin seemed to be affected by the charge of the two reactants and at high doses not all adsorbate was released. PS4A, because of structural and compositional differences, had to be loaded on to the chitosan by freeze drying a suspension of the nanoparticles in a solution of the drug. After a rapid (burst) release phase, the rate of release into water was steady for the next 4 h, but not all the drug was released. In-vivo it was evident that PS4A and PS4α were equally active in solution or when formulated in the chitosan nanoparticles.

These results show that chitosan nanoparticles, readily prepared without the use of organic solvents, are a suitable vehicle for the delivery of these immunostimulants from *M. vaccae*; the formulations might find application as antitumour agents.

Mycobacterium vaccae is a rapidly growing mycobacterium that, like a number of similar organisms, is capable of acting as an immunostimulant. Recently, heat-sterilized suspensions of this organism have been used clinically in the treatment of tuberculosis (Stanford & Stanford 1994) and have been explored as vaccine adjuvants or in the treatment of cancer and asthma (Scrip 1998). Recent reports of clinical activity have suggested a less than ideal response (Scrip 1997) and it seemed possible that isolated and purified active components might offer a better and more reproducible immunoresponse. By analogy with the isolation of immunostimulants from Bacillus Calmette–Guérin (BCG vaccine), an attenuated *M. bovis*, (Wang et al 1995), we have reported the isolation and biological activity of PS4, an active component of *M. vaccae* (Groves et al 1995). Further work has shown that the active component of this mixed material is a proteoglycan with a 20-kDa protein backbone to which are attached short ($\sim$4 kDa) glucans and an *O*-methylpentopyranoside (Tian et al, unpublished results). The overall molecular weight of the component separated by

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extraction with boiling water (PS4A) is approximately 50 kDa, although this is increased to approximately 150 kDa by self-association. Separation in the cold with 8 M urea produced a structurally similar material (PS4α) but with a molecular weight of approximately 20 MDa. Both materials have considerable biological activity against a murine S-180 sarcoma in-vivo but, like the BCG isolates, had no in-vitro activity against a number of isolated cancer cells. This suggested an indirect mode of action involving stimulation of macrophages. Formulation has therefore become an issue, because it would be desirable to prolong macrophage stimulation and assist in the presentation of these complex molecules if they were to be effective as adjuvants. With PS1, which has a similar mode of action, macrophage stimulation resulted in rapid destruction of both gelatin and collagen vehicles (Friess et al 1996). This observation was the basis for the use of hydroxymethylcellulose formulations against a colon cancer (Caco-2) in man (Ciftci et al 1996). However, we have observed that chitosan nanoparticles might be a better vehicle for delivery of these immunostimulants.

Chitosan is a biocompatible and biodegradable natural biopolymer consisting of β-1$\rightarrow$4 linked 2-amino-2-deoxyglucopyranose. It is currently manufactured commercially on a large scale by alkaline N-deacetylation of chitin, an abundant biopolymer isolated from the outer shells of crustaceans such as crabs and shrimps. Chitosan has rather specific solution properties. It is only soluble at acidic pH when the free amino group ($-NH_2$) becomes protonated to form a cationic amine group ($-NH_3^+$). At these pHs chitosan has been identified as a linear polycation that readily adheres to negatively charged surfaces, such as skin, mucus and proteins (Lehr et al 1992; Calvo et al 1997). Recent studies indicate that the binding of chitosan to epithelial membranes resulted in cellular F-actin depolymerization and disbandment of the tight junction protein ZO-1. This property is currently considered to be the major mechanism by which chitosan enhances drug adsorption (Schipper et al 1997). In-vivo, chitosan is degraded by several glycosidases, primarily lysozyme and *N*-acetylglucosaminidase (Muzzarelli 1997). Chitosan also has stimulatory effects on macrophages and this activity was suggested as being mainly attributable to the remaining *N*-acetylglucosamine groups (Peluso et al 1994). Activated macrophages were found to be able to accelerate the in-vivo degradation of chitosan (Muzzarelli 1997). The availability of chitosan, its biocompatibility and its unique chemical and biological properties made it an attractive biomaterial for a variety of pharmaceutical applications, especially in the areas of wound dressing and drug delivery. It has been used or tested in different forms, such as tablets, matrix and microparticles for the purpose of sustained release (Sawayanagi et al 1982; Shiraishi et al 1993; Jameela & Jayakrishnan 1995; Hari et al 1996), controlled drug delivery (Hassan et al 1992), mucosal formulations (Illum et al 1996; Aspden et al 1997) and, more recently, drug-absorption enhancement (Luessen et al 1996), protein and peptide drug delivery and vaccine development (Elcin et al 1996; Calvo et al 1997; Liu et al 1997). The low solubility of high-molecular-weight chitosan at normal physiological pH has, to some extent, limited the application of this otherwise attractive biopolymer. Conversion of chitosan into microparticles could be an ideal way to overcome this problem. Chitosan microparticles prepared by different emulsion-based techniques, such as solvent extraction or solvent evaporation, have been reported (Thanoo et al 1992; Akbuga & Bergisadi 1996). The emulsion techniques use organic solvents and strong mechanical forces, which would be problematical for the stability of many incorporated biological molecules. Recent studies have revealed that chitosan nanoparticles could be formed spontaneously as a result of ionic interaction between the positively charged amino group and negatively charged multivalent counter-ions, such as tripolyphosphate and sulphate groups. Novel precipitation methods based on this unique property of chitosan have been developed and have proved efficient in preparing chitosan nanoparticles (Berthold et al 1996; Calvo et al 1997). The attractive features of these techniques include the spontaneous formation of chitosan nanoparticles by use of a relatively mild process, the homogenous and adjustable resultant particle size, and there was no need for organic solvents (Calvo et al 1997).

Materials and Methods

Bacteria and media

Mycobacterium vaccae (ATCC #15483) was cultivated in tryptic soy broth (TSB, Gibco Laboratories, Grand Island, NJ) at 37°C for 5 to 7 days. The bacteria were collected by centrifugation at 10 000 rev min^{-1} for 20 min with a Sorval RC-5B refrigerated superspeed centrifuge (Du Pont, Newtown, CT) and washed twice with double-distilled water. Washed cells were lyophilized by use of a Labconco Lyph-lock 4·5 freeze-drying system (Labconco Corporation, Kansas City, MO) to determine the dry weight.

Chitosan (practical grade, 75–85% deacetylated, molecular weight $\sim$150 kDa) was from Aldrich (Milwaukee, WI); endotoxins were not tested.

Bovine serum albumin (BSA) was from Intergen (Purchase, NY), BCA protein assay reagent and BSA standard from Pierce (Rockford, IL), protein assay reagent from Bio-Rad (Hercules, CA), sodium sulphate and phosphate-buffered saline (PBS, pH 7·4) from Sigma (St Louis, MO), and mannitol from Fluka (Ronkonkoma, NY). Other reagents were obtained from Fisher Scientific (Itasca, IL).

Extraction of the proteoglycans

By boiling. Lyophilized *M. vaccae* cells ($\sim$0·5 g) were extracted by heating under reflux with double-distilled water (500 mL) for 2 h (Lou 1993). The extract was clarified by passage through a 0·2-μm porous membrane (Millipore Corporation, Bedford, MA) and concentrated to 10 mL (approx.) under vacuum at 65°C by rotary evaporation (Büchi Rotavapor; Brinckman Instruments, Westbury, NY). The concentrated extract was dialysed overnight against double-distilled water and lyophilized for storage. This material was identified as PS4A.

With urea. Lyophilized *M. vaccae* cells ($\sim$0·5 g) were added to an aqueous solution of urea (8 M, 200 mL) and stirred for up to 72 h at room temperature. The mixture was clarified by filtration through a 0·2-μm membrane and then dialysis against double-distilled water for 48 h. Concentration and lyophilization were conducted as described above. The urea-extracted material was identified as PS4α.

Preparation of chitosan nanoparticles

Chitosan nanoparticles were prepared by an adaptation of the method described by Berthold et al (1996). Chitosan (1·25 g) was dissolved in acetic acid (2% (v/v), 500 mL) containing 1% (v/v) Tween 80. The solution was transferred to a sonication bath (Solid State Ultrasonic FS-9, Fisher Scientific, Itasca, IL) and stirred at low speed ($\sim$30% of maximum speed) with a blade stirrer (Stedfast Stirrer, Model SL2400, Fisher Scientific). Sodium sulphate solution (20%, w/v) was added dropwise during sonication with stirring to a final sodium sulphate concentration of 0·5% (w/v, approx.). Sonication and stirring were continued for 30 min. Glutaraldehyde solution (25%, 5·0 mL) was added and sonication and stirring were continued for another hour when cross-linking was stopped by addition of sodium metabisulphite solution (12% (w/v), 100 mL). Particle-size changes during the preparation process were monitored by photon correlation spectroscopy (PCS). The nanoparticles formed were finally recovered by centrifugation at 5000 rev min^{-1} for 15 min with a Sorval RC-5B refrigerated superspeed centrifuge (Dupont) and washed twice with double-distilled water. Recovered nanoparticles were suspended in double-distilled water (500 mL). A small portion of the total suspension (20 mL) was lyophilized to determine the total mass of recovered particles. The chitosan nanoparticles could be kept as a suspension in water for at least 1 month at 4°C or were lyophilized by diluting with double-distilled water and dividing into 5-mL portions containing 2% (w/v) of D-mannitol as a cryopreservative agent.

Particle size was measured by PCS with a Malvern Zetasizer 3 particle electrophoresis and multi-angle particle-size analyser (Malvern Instruments, Southborough, MA). This equipment was equipped with an AZ10 sizing cell and a 5-mW helium-neon laser light source (633 nm) operated at 90° to the detector. The signals were processed by a Malvern 7032N/72C Multi-8-bit 72-channel digital correlator. The particle size distribution was also examined by scanning electron microscopy (SEM). For SEM examination several drops of diluted nanoparticle suspension were added to a 0·2-μm filter membrane. The membrane was air-dried overnight and mounted with adhesive on to an aluminium peg (Electron Microscopy Science, Ft. Washington, PA). The sample was spattered with gold-palladium under vacuum and observed with a Jeol J35 scanning electron microscope. The surface charge of nanoparticles in water or PBS (pH 7·4) was measured with a Lazer Zee Meter Model 510 (Pen Kem, Badford Hill, NY).

Adsorption of bovine serum albumin (BSA)

BSA stock solution (40 mg mL^{-1}) was prepared with double-distilled water. A chitosan nanoparticle suspension was accurately distributed into 20 vials (5 mL each) and four vials were lyophilized to constant weight to determine the mass of the chitosan nanoparticles/vial. BSA solutions, 50–4000 μg mL^{-1}, were added and shaken (100 rev min^{-1}) at room temperature for up to 3 h using an orbit shaker (Lab-Line Instruments, Melrose Park, IL). Protein solution without chitosan nanoparticles was used as control. Nanoparticles were removed by centrifugation at 7000 rev min^{-1} for 10 min and the supernatant recovered for protein assay by use of the commercial BCA method. The amount of loaded protein was calculated from the difference between results from loaded media and from the control solution and expressed as microgram (μg) protein/milligram (mg) chitosan nanoparticles. The data fitted a Langmuir isotherm (Figure 1). The loading efficiency was defined as

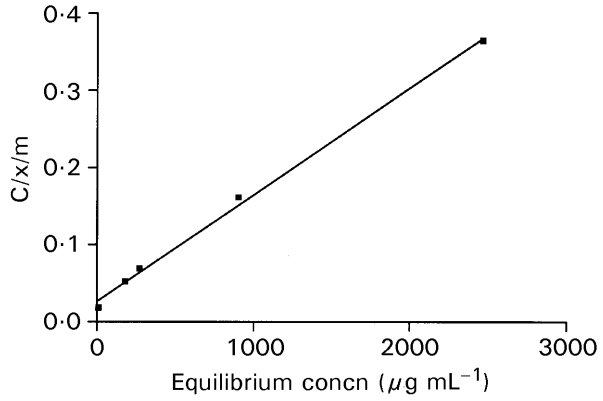

Figure 1. Langmuir isotherm of adsorption of BSA by chitosan nanoparticles ($r^2 = 0{\cdot}997$). (C = equilibrium concentration, $\mu g\,mL^{-1}$; x = amount (μg) of BSA adsorbed; m = mass (mg) of chitosan nanoparticles).

$(1 - C_f/C_o) \times 100\%$, where C_f and C_o were the concentrations of BSA in the final loading media and the initial or control solutions, respectively.

BSA release

BSA-loaded chitosan nanoparticles were prepared by adding BSA to a nanoparticle suspension ($\sim 276\,\mu g\,mL^{-1}$); final BSA concentrations were 50 and $200\,\mu g\,mL^{-1}$. Because it had previously been determined that the loading efficiency at these BSA concentrations was close to 100%, no separation was conducted. The BSA-nanoparticle mixture was directly lyophilized in 5-mL portions containing 2% (w/v) D-mannitol after shaking at room temperature for 2 h. One vial of the prepared particles was resuspended in PBS (pH 7·4, 1·0 mL) containing 0·02% sodium azide and incubated at 37°C with constant shaking in a Precision Model 25 reciprocal shaking bath (Precision Scientifics, Chicago, IL). The sample was centrifuged at $7000\,rev\,min^{-1}$ for 10 min and supernatant (0·8 mL) was taken for protein assay. The removed supernatant was replaced with fresh PBS (0·8 mL) and the sampling process repeated at 30-min intervals until no more protein could be detected in the supernatant. The experiment was duplicated. The release data were initially presented by plotting the cumulative amount (mass) released against time followed by calculation and comparison with the release rates at different time intervals for a unit amount of nanoparticles (Lou 1993).

Loading of PS4A (or PS4α) on to chitosan nanoparticles

PS4A loading was conducted by direct lyophilization of chitosan nanoparticles in water containing the required amount of compound. PS4A samples were first dissolved in double-distilled water (target concentration $2\,mg\,mL^{-1}$). The protein and polysaccharide content of the solution were determined by means of the Bio-Rad protein assay and phenol sulphuric acid assay, respectively. The PS4A or PS4α solutions were mixed with an equal volume of a suspension containing chitosan nanoparticles ($\sim 300\,\mu g\,mL^{-1}$). The mixture was kept at room temperature under constant shaking for 1 h and then lyophilized in 2-mL portions to which 2% (w/v) D-mannitol was added. To determine the loading efficiency, two vials of lyophilized formulation were resuspended in water (2 mL) and immediately filtered through 0·2-μm Acrodisc syringe-filters (Gelman Sciences, Ann Arbor, MI). The protein content of the filtrate was assayed by the Bio-Rad protein assay and the amount of PS4A loaded was calculated. The release of PS4A was measured and analysed by the same procedures. Briefly, the contents of one vial of the PS4A formulation were reconstituted in PBS (pH 7·4, 1·5 mL) containing 0·02% sodium azide and incubated at 37°C with constant shaking. After 30 min the samples were centrifuged at $7000\,rev\,min^{-1}$ for 10 min and supernatant (1·0 mL) was taken for protein assay. The removed supernatant was replaced with fresh PBS (1·0 mL) and the particles resuspended. This process was repeated at 30-min intervals until no more protein could be detected in the supernatant.

In-vivo S-180 test

The antineoplastic activity of PS4A formulations was tested by use of an S-180 murine sarcoma model (Klegerman et al 1991). The formulation was sterilized for 12 h with ethylene oxide gas at the University of Illinois Hospital. One vial of formulation (containing 2 mg PS4A and $\sim 150\,\mu g$ chitosan nanoparticles) was suspended in PBS (2 mL) and diluted to the desired concentration. The suspension was mixed with an equal volume of $3 \times 10^6\,mL^{-1}$ viable S-180 sarcoma cell suspension. Each mouse was injected subcutaneously with mixed suspension containing 3×10^5 S-180 sarcoma cells and the PS4A or PS4α formulation (0·2 mL). After 14 days, the mice were killed, dissected and tumour incidence and tumour volume were determined (Klegerman et al 1991).

Results and Discussion

Without glutaraldehyde fixation the nascent chitosan nanoparticles proved to be unstable and could not resist the washing process. Cross-linked, the particles stabilized with diameters measured by

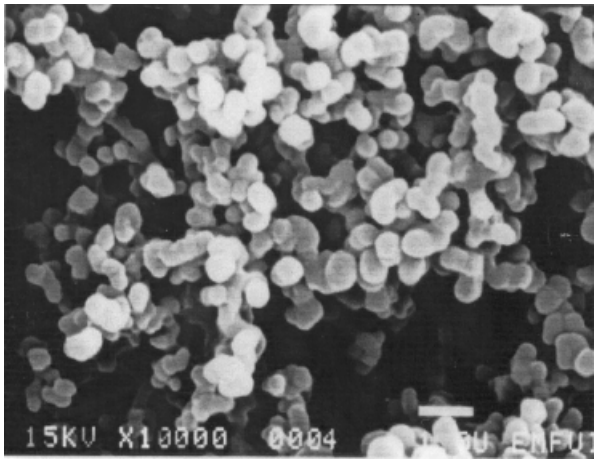

Figure 2. Scanning electron microscopy (SEM) of chitosan nanoparticles (magnification × 10 000; the bar is 1 μm long).

PCS of 700 ± 100 nm and by SEM of ~600 nm (Figure 2), suggesting that the chitosan matrix structure was analogous to that of a sponge, shrinking on drying. In water the nanoparticles were positively charged (+37·7 ± 1·9 mV); in PBS at pH 7·4 the charge was lower (+10 ± 0·6 mV). Loading with BSA (MW ~69 kDa, pI 4·8) was rapid and efficient but for loadings above 800 μg mL^{-1} the overall charge of the nanoparticles (−18 mV ± 0·9) was reversed and the colloidal suspension became aggregated and flocculated. The BSA adsorption process fitted a Langmuir isotherm (Figure 1).

In PBS, the BSA concentration equilibrated rapidly (1 h at 37°C). A dissolution study indicated a slow-release phase for up to 5 h (Figure 3). By correcting for the mass of drug released in the first hour, the so-called burst effect, it is possible to calculate a steady-state release rate (Lou 1993) (Table 1). Although almost 100% of the BSA was released when loaded at 250 μg BSA/1·38 mg chitosan, at the dose of 1000 μg/1·38 mg only 35% (approx.) of the total protein was released (Figure 3), suggesting that the remainder was trapped within the matrix. In this situation most of the incorporated drug would be released by degradation or by erosion of the matrix and this would provide a long-term sustained-release effect which would only be likely to be manifest in-vivo.

Although PS4A molecules have a pI (~6·6) similar to that of BSA, the loading efficiency (~60 ± 10%) was lower than that of BSA (100%), probably because of the different structure and composition. To incorporate PS4A and PS4α it was necessary to suspend the chitosan nanoparticles in aqueous solutions of the drugs, and then freeze-dry.

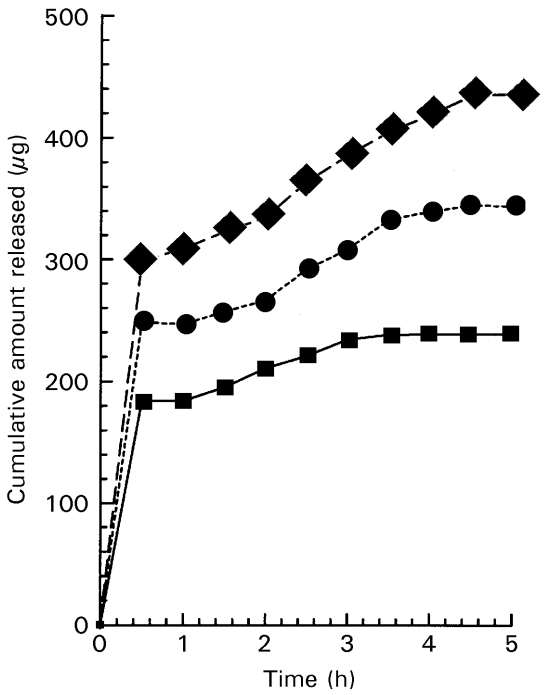

Figure 3. Release of BSA into PBS at pH 7·4 and 37°C (◆, 1000 μg; ●, 500 μg; ■, 250 μg).

Table 1. Rate of release of bovine serum albumin into phosphate-buffered saline.

Time (h) loading:	Rate of release (μg/mg particles h^{-1}) from chitosan particles of		
	250 μg/ 1·38 mg	500 μg/ 1·38 mg	1000 μg/ 1·38 mg
1·5	22·5	21·5	29
2·0	31	18	23
2·5	19	51	58
3·0	22	27	34
3·5	8	49	34
4·0	–	14	28
4·5	–	10	32
5·0	–	–	4

The weak binding promoted rapid release of PS4A over the first 30 min but beyond that time the release rate settled to a steady state over the next 4–5 h (Figure 4), suggesting controlled diffusional release from the interior of the swollen matrix of the carrier nanoparticles.

Within the confines of inherently variable biological data it is evident from Tables 2 and 3 that the in-vivo antitumour activity of PS4A and PS4α and their respective formulations were similar at the 100 μg/mouse (5 mg kg^{-1}) level.

We conclude that chitosan nanoparticles, readily prepared without the use of organic solvents, are a suitable vehicle for the delivery of these

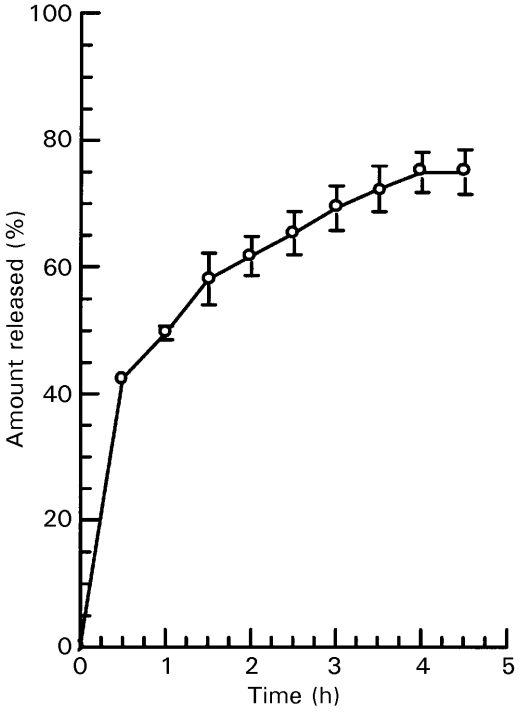

Figure 4. Release of PS4A from chitosan nanoparticles (n = 4, mean ± standard deviation).

Table 2. Antineoplastic activity of PS4A formulations.

Group	Number of animals	Tumour incidence	P^a
Control	10	0·6 (6/10)	–
Chitosan particles	10	0·3 (3/10)	0·185
PS4A (100 μg)	10	0·2 (2/10)	0·09
PS4A (10 μg)	10	0·5 (5/10)	0·815
Chitosan + PS4A (100 μg)	10	0·1 (1/10)	0·029
Chitosan + PS4A (10 μg)	10	0·4 (4/10)	0·328

[a]P value obtained by χ^2 analysis of the tumour incidence data.

Table 3. Antineoplastic activity of PS4α formulations.

Group	Number of animals	Tumour incidence	P^a
Control	9	0·78 (7/9)	–
Chitosan particles	10	1·0 (10/10)	–
PS4α (100 μg)	10	0·2 (2/10)	0·012
PS4α (10 μg)	10	0·3 (3/10)	0·185
Chitosan + PS4α (100 μg)	10	0·2 (2/10)	0·012
Chitosan + PS4α (10 μg)	10	0·2 (2/10)	0·012

[a]P value obtained by χ^2 analysis of the tumour incidence data.

immuno-stimulants obtained from *M. vaccae*. The formulations might find application as antitumour agents.

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References

Akbuga, J., Bergisadi, N. (1996) 5-fluorouracil-loaded chitosan microspheres: preparation and release characteristics. J. Microencap. 13: 161–168

Aspden, T. J., Mason, J. D. T., Jones, N. S., Lowe, J., Skaugrud, Ø., Illum, L. (1997) Chitosan as a nasal delivery system: the effect of chitosan solutions on in-vitro and in-vivo mucociliary transport rates in human turbinates and volunteers. J. Pharm. Sci. 86: 509–513

Berthold, A., Cremer, K., Kreuter, J. (1996) Preparation and characterization of chitosan microspheres as drug carrier for prednisolone sodium phosphate as model for anti-inflammatory drugs. J. Contr. Rel. 39: 17–25

Calvo, P., Remuñan-López, C., Vila-Jato, J. L., Alonso, M. J. (1997) Chitosan and chitosan/ethylene oxide-propylene oxide block copolymer nanoparticles as novel carriers for proteins and vaccines. Pharm. Res. 14: 1431–1436

Ciftci, K., Klegerman, M. E., Tian, X. X., Groves, M. J. (1996) In-vivo testing of coated hydroxypropylmethy-cellulose granules containing an antineoplastic glycan (PS4) using Caco-2 tumour cells. Pharm. Sci. 2: 357–360

Elcin, Y. M., Dixit, V., Gitnick, G. (1996) Controlled release of endothelial cell growth factor from chitosan-albumin microspheres for localized angiogenesis: in-vitro and in-vivo studies. Art. Cell. Blood Subst. Immobil. Biotech. 24: 257–271

Friess, W., Zhou, W., Groves, M. J. (1996) In-vivo testing of collagen matrices containing PS1, an antineoplastic glycan against murine sarcoma cells. Pharm. Sci. 2: 121–124

Groves, M. J., Klegerman, M. E., Ciftci, K., Tian, X. X. (1995) Isolation, characterization and formulation of an antineoplastic glycan from cultures of *Mycobacterium vaccae*. J. Pharm. Pharmacol. 47: 1082

Hari, P. R., Chandy, T., Sharma, C. P. (1996) Chitosan/calcium alginate microcapsules for intestinal delivery of nitrofurantoin. J. Microencap. 13: 319–329

Hassan, E. E., Parish, R. C., Gallo, J. M. (1992) Optimized formulation of magnetic chitosan microspheres containing the anticancer agent, oxantrazole. Pharm. Res. 9: 390–397

Illum, L., Davis, S. S., Pawula, M., Fisher, A. N., Barrett, D. A., Farraj, N. F., Shaw, P. N. (1996) Nasal administration of morphine-6-glucuronide in sheep—a pharmacokinetics study. Biopharm. Drug Disp. 17: 717–724

Jameela, S. R., Jayakrishnan, A. (1995) Glutaraldehyde cross-linked chitosan microspheres as a long acting biodegradable drug delivery vehicle: studies on the in vivo degradation of microspheres in rat muscle. Biomaterials 16: 769–775

Klegerman, M. E., Ujjainwala, L., Zeunert, P. (1991) High-dose inhibition and low-dose enhancement of murine sarcoma growth exhibited by BCG vaccine. Cancer Lett. 56: 137–145

Lehr, C. M., Bouwstra, J. A., Schacht, E. H., Juninger, H. E. (1992) In vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers. Int. J. Pharm. 78: 43–48

Liu, L.-S., Liu, S.-Q., Ng, S. Y., Froix, M., Ohno, T., Heller, J. (1997) Controlled release of interleukin-2 for tumour immunotherapy using alginate/chitosan porous microspheres. J. Contr. Rel. 43: 65–74

Lou, Y. (1993) The isolation, properties and formulation of an antineoplastic agent derived from Bacillus Calmette-Guérine. Ph. D. thesis, University of Illinois at Chicago, Chicago, IL, pp 99–131

Luessen, H. L., de Leeuw, B. J., Langemeyer, M. W., de Boer, A. B., Verhoef, J. C., Junginger, H. E. (1996) Mucoadhesive polymers in peroral peptide drug delivery. VI. Carbomer and chitosan improve the intestinal absorption of the peptide drug buserelin in-vivo. Pharm. Res. 13: 1668–1672

Muzzarelli, R. A. (1997) Human enzymatic activities related to the therapeutic administration of chitin derivatives. Cell. Mol. Life Sci. 53: 131–140

Peluso, G., Patella, O., Rainier, M., Satin, M., Ambrosia, L., Celebre, D., Avalon, B., Balsam, G. (1994) Chitosan-mediated stimulation of macrophage function. Biomaterials 15: 1215–1220

Sawayanagi, Y., Nambu, N., Nagai, T. (1982) Use of chitosan for sustained-release preparations of water-soluble drugs. Chem. Pharm. Bull. 30: 4213–4215

Schipper, N. G. M., Olsson, S., Hoogstraate, J. A., DeBoer, A. G., Vårum, K. M., Artursson, P. (1997) Chitosans as absorption enhancers for poorly absorbable drugs 2: mechanism of absorption enhancement. Pharm. Res. 14: 923–929

Scrip (1997) Stanford Rook's TB therapy founders. No. 2273, p. 23

Scrip (1998) Stanford Rook/Onyvax sign cancer vaccine deal. No. 2321, p. 9

Shiraishi, S., Imai, T., Otagiri, M. (1993) Controlled release of indomethacin by chitosan-polyelectrolyte complex: optimization and in-vivo/in-vitro evaluation. J. Contr. Rel. 25: 217–225

Stanford, J. L., Stanford, C. A. (1994) Immunotherapy of tuberculosis with *Mycobacterium vaccae* NCTC 11659. Immunology 191: 555–563

Thanoo, B. C., Sunny, M. C., Jayakrishnan, A. (1992) Cross-linked chitosan microspheres: preparation and evaluation as a matrix for the controlled release of pharmaceuticals. J. Pharm. Pharmacol. 44: 283–286

Wang, R., Klegerman, M. E., Marsden, I., Sinnott, M., Groves, M. J. (1995) An antineoplastic glycan isolated from *Mycobacterium bovis* (BCG vaccine). Biochem. J. 311: 867–872