ELSEVIER

International Journal of Pharmaceutics 128 (1996) 99–111

international journal of pharmaceutics

Evaluation of *N*-(2-hydroxypropyl)methacrylamide copolymer-peptide conjugates as potential oral vaccines. Studies on their degradation by isolated rat small intestinal peptidases and their uptake by adult rat small intestinal tissue in vitro

Suzanne M. Morgan[a], Vladimir Subr[b], Karel Ulbrich[b], John F. Woodley[c], Ruth Duncan[d,*]

[a]*CRC Institute for Cancer Studies, The Medical School, University of Birmingham, Birmingham, B15 2TJ, UK*
[b]*Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic*
[c]*Drug Delivery Group, Keele University, Keele, Staffordshire, ST5 5BG, UK*
[d]*Centre for Polymer Therapeutics, The School of Pharmacy, 29–39 Brunswick Square, London, WC1N 1AX, UK*

Received 1 March 1995; accepted 12 June 1995

Abstract

Oral administration of therapeutic peptides and peptide antigens has achieved limited success owing to their degradation and poor transport across the gastrointestinal tract. In this study covalent coupling of peptides to the water soluble polymer *N*-(2-hydroxypropyl)methacrylamide (HPMA) is explored as a means to overcome these problems. A model peptide, b-chain of insulin (b-chain), and the human rhinovirus antigenic determinant, peptide VP2, were covalently bound to HPMA copolymers of molecular weight 23 200 to give a peptide content of approximately 25% (w/w). Conjugation resulted in a marked reduction in the rate of degradation of both peptides during in vitro incubation with small intestinal brush border (BBM) and luminal enzymes. In the case of b-chain, reductions of up to 80% and 60% were observed with BBM and luminal peptidases, respectively. For peptide VP2, reductions up to a maximum of 80% and 55% were observed with BBM and luminal peptidases, respectively. Incubation of ^{125}I-labelled b-chain with everted rat jejunal sacs in vitro showed no serosal transfer of intact free ^{125}I-labelled b-chain as a result of peptide degradation. In contrast, the ^{125}I-labelled HPMA copolymer-peptide conjugate displayed transfer of intact b-chain into the serosal fluid, and sacs with or without Peyer's Patches (PP) displayed transfer of 66 and 58 ng of conjugated b-chain per mg tissue protein. As polymer conjugation both protects against peptide degradation and promotes peptide uptake, HPMA copolymer conjugation has the potential to improve oral vaccination using peptide antigens.

Keywords: *N*-(2-Hydroxypropyl)methacrylamide copolymers; Peptide vaccines; Oral drug delivery

* Corresponding author. Tel.: +44 171 753 5932; fax: +44 171 753 5931.

SSDI 0378-5173(95)04228-3

1. Introduction

Recent advances in biotechnology have generated a large number of peptide drugs, and in particular peptide sequences that might provide useful antigens for immunisation. Administration of such compounds is generally restricted to the parenteral route, with the obvious drawbacks associated with administration by injection. Conceptually oral administration would be the most convenient route for antigen delivery. Indeed, based on the concept of the common mucosal immune system through which antigen activated T helper (Th) cells and B cells from the gut associated lymphoid tissue disseminate to other mucosal tissues such as the bronchial associated lymphoid tissue (reviewed by Marshall et al., 1989), it can offer a practical solution to development of vaccines against infections in mucosal sites such as the respiratory tract. It is envisaged that the low systemic bioavailability of peptides following oral administration due to extensive proteolysis, both in the gastrointestinal lumen and at the BBM (Woodley, 1991), and their slow rate of transport across the intestinal mucosa (Cartlidge et al., 1986) would limit both the efficiency and reproducibility of oral vaccination. Recent studies have examined the possibility of using drug delivery systems such as liposomes (Genco et al., 1983) and particles (Eldridge et al., 1989, 1990; O'Hagan et al., 1989; McGee et al., 1994; Maloy et al., 1994) to facilitate oral administration of antigens. For example, oral administration of a toxoid vaccine of staphylococcus enterotoxin B in poly[DL-lactide-co-glycolide] microspheres induced a disseminated mucosal IgA antitoxin antibody response (Eldridge et al., 1990).

Here HPMA copolymer-peptide conjugates were examined to investigate whether they might prove useful to overcome the problems associated with proteolytic degradation of peptide antigens and also to facilitate serosal transfer. HPMA copolymers are inert, biocompatible polymers under development as a targetable drug delivery system for systemic administration; conjugates containing the anti-tumour agent doxorubicin are currently undergoing clinical investigation (reviewed by Duncan, 1992). We have shown previously that antibodies covalently bound to HPMA copolymers are protected against degradation by lysosomal enzymes (Seymour et al., 1991). Furthermore, conjugation of HPMA to an antibody afforded its protection against degradation by BBM enzymes (Al-Shamkhani and Duncan, unpublished data). It has been shown that soluble HPMA copolymers are transported from the mucosal to the serosal side of adult everted gut sacs (Cartlidge et al., 1986). Such knowledge about HPMA copolymers led us to consider using this polymer to facilitate pinocytic uptake and transcytosis of covalently coupled peptide vaccines by small intestinal enterocytes and M cells.

HPMA copolymer conjugates were synthesised which contained b-chain of insulin (as a model compound), or the peptide VP2 derived from human rhinovirus (Francis et al., 1989). This peptide antigen is a construct containing B cell and Th cell epitopes derived from two sites on the structural protein VP2 of human rhinovirus type 2 (amino acids 156–170 and 24–33, respectively). The stability of HPMA copolymer-b chain and HPMA copolymer-peptide VP2 conjugates were measured in the presence of rat small intestinal luminal and BBM peptidases, and the uptake of ^{125}I-labelled b-chain and a ^{125}I-labelled HPMA-b chain conjugate by adult rat small intestinal tissue in vitro was also assessed using an everted rat jejunal organ culture method (Bridges et al., 1978; Bridges, 1980).

2. Materials and methods

2.1. Preparation of b-chain

The b-chain used in these studies had the following amino acid sequence (FVNEHLCGSHLVEALYLVCGERGFFYTPKA) and was prepared from human insulin (supplied by CP Pharmaceuticals Ltd., Wrexham, UK) using the method of Crestfield et al. (1963).

2.2. Synthesis of peptide VP2

Peptide VP2 had the following amino acid sequence (VKAETRLNPDLQPTETSQDVANAIVC) and was prepared by solid-phase peptide synthesis according to the Merrifield (1963) procedure. The B cell epitope was at the amino terminus, and the peptide had a non-natural cysteine residue at the carboxyl terminus.

2.3. Preparation and characterisation of HPMA copolymer-peptide conjugates

Methacryloylchloride (freshly distilled before use), 1-amino-2-propanol, glycylglycine, *p*-nitrophenol, azo-bis-isobutyronitrile (AIBN) and DCCI were supplied by Fluka A.G., Switzerland.

First, HPMA copolymer containing 4.58 mol% glycylglycine-*p*-nitrophenol side-chains was synthesised as previously described (Rihova and Kopecek, 1985) (Fig. 1). Briefly, HPMA monomer was prepared as described by Strohalm and Kopecek (1978). Methacryloylglycylglycine *p*-nitrophenyl-ester was prepared by methacryloylation of glycylglycine, subsequently bound to *p*-nitrophenol using dicyclohexylcarbodiimide (DCCI). The HPMA copolymer (polymeric precursor) was then prepared by radical precipitation copolymerisation of HPMA with methacryloylglycylglycine *p*-nitrophenyl-ester (5 mol%) in acetone using AIBN as initiator. The resulting reactive polymeric precursor had a weight average molecular weight (Mw) of 23 200 and a polydispersity (Mw/Mn; where Mn = number average molecular weight) of 1.33 as estimated by gel permeation chromatography (GPC) after aminolysis of the precursor with an excess of 1-amino-2-propanol. GPC analysis was carried out using a column of sepharose 4B and 6B (1:1; 100 mm × 13 mm) and eluted with Tris–HCl buffer (0.02 M; pH 8.0) containing NaCl (0.5 M) at a flow rate of 0.2 ml/min, and detection was performed with a refractive index detector.

To prepare the HPMA copolymer-peptide conjugates, HPMA-glycyl-glycine-*p*-nitrophenol (195 mg) and the peptide (62 mg) were separately dissolved in NaCl (3 ml; 0.15 M), and the peptide solution was added to the polymer solution on ice, the reaction having a molar excess of *p*-nitrophenol groups relative to the peptide primary amine groups. The reaction was left for 7 h, and was buffered throughout (pH 7.5–8.2) by the addition of 20-μl aliquots of a saturated sodium borate solution. Then, 300 μl of the sodium borate solution was added and the reaction mixture was left at 4°C for a further 16 h. Finally, any remaining *p*-nitrophenol ester groups were aminolysed by the addition of excess aminopropanol (100 μl; 1 M). Free *p*-nitrophenol was removed from the reaction mixture by dialysis and the product was lyophilised.

The Mw of the resulting product was analysed by size-exclusion HPLC using a TSK G 3000 PW analytical column (7.5 mm × 300 mm) eluted with phosphate buffer saline containing NaCl (0.4 M) at a flow rate of 0.75 ml/min and with a variable wavelength UV detector (280 nm). The column was calibrated using HPMA polymer standards of defined Mw, and the amount of conjugated peptide was determined by UV absorbance (280 nm).

2.4. Isolation of rat small intestinal BBM and luminal enzymes

BBM enzymes were isolated from rat small intestine as previously described (Kessler et al., 1978). Male Wister rats (2–4 animals, 300–350 g) were starved overnight, then sacrificed and their small intestine was removed, washed with ice-cold PBS (to give luminal washings) and then cooled on ice. Briefly, the small intestinal mucosa, obtained by scraping the tissue with a glass slide, was suspended in a Tris–HCl buffer (0.02 M, pH 7.4, containing 0.05 M mannitol and 0.02 mM phenylmethylsulphonylfluoride) and homogenised. $CaCl_2$ (0.01 M) was added and the homogenate was spun (3000 × *g*, 4°C for 30 min), and to obtain a BBM pellet the resulting supernatant was spun at 25 000 × *g* (30 min; 4°C). Following resuspension in the buffer, aliquots (100 μl) were frozen in liquid N_2 and stored at −70°C.

To isolate the luminal enzymes (Woodley, 1991), the luminal washings, obtained as described above, were suspended in a blender and

filtered through glass wool to remove coarse particles. The suspension was centrifuged (1500 × g, 4°C) for 15 min. The pellet was resuspended in PBS (10 ml) and dispensed into aliquots (0.5 ml), frozen in liquid N_2 and stored at −70°C. The total protein content of the enzyme samples was estimated by the Lowry protein assay (Lowry et al., 1951) as modified by Peterson (1983).

2.5. Degradation of peptides and HPMA-peptide conjugates by small intestinal peptidases

The degradation of free peptide and the HPMA copolymer-peptide conjugates was assessed by incubation of different concentrations of each substrate (based on peptide concentration) with a fixed concentration of either BBM or luminal enzymes. To obtain initial rates of degradation, analysis was carried out at several time points for each substrate concentration. Substrate and enzyme were diluted in Tris–HCl buffer (0.02 M, pH 7.4), additionally containing 6.67 mM $CaCl_2$ for the luminal enzyme studies (Britton, 1989). Typically, 270 μl of substrate and 75 μl of H_20 were pre-incubated for 5 min at 37°C. Then, 30 μl of BBM or luminal preparation (equivalent to 4.5 μg protein of each preparation for b-chain and 90 μg BBM protein or 200 μg luminal protein for peptide VP2) was added, the mixture vortexed and re-incubated at 37°C. Incubations were also set up without enzyme or without substrate, to act as controls for this study. Samples (50 μl) were withdrawn at intervals and dispensed into LP3 tubes containing 1 M HCl (5 μl), frozen immediately and stored at −70°C until analysis by reverse phase (RP) HPLC. On analysis, aliquots (30 μl) of each sample were injected onto a Kromasil 100 C_{18} column (4.5 mm × 150 mm) and eluted using a linear gradient of 5–45% acetonitrile/H_20; 0.1% TFA over 20 min. A variable wavelength UV detector (214 nm) was used for detection.

2.6. Everted gut sac model for uptake in vitro

^{125}I-Labelled b-chain and ^{125}I-labelled HPMA-b chain were prepared using the chloramine T method (McFarlane, 1958). Free [^{125}I]iodide was removed by dialysis against 1% (w/v) NaCl. Radiolabelling efficiency was estimated using paper electrophoresis (Whatman No.1 filter paper, run in sodium barbitone buffer (0.05 M, pH 8.6) for 25 min at 400 mV, 10–15 Ma). The specific activity of the labelled products was approximately 0.25 mCi/mg.

Everted small intestinal gut sacs were prepared using an established method (Bridges et al., 1978, Bridges, 1980). Briefly, adult male Wister rats (250–300 g) were starved for 24 h, sacrificed and the small intestine excised and washed with oxygenated TC medium 199 (37°C). The small intestine was everted on a notched glass rod, secured with an artery clamp at one end, filled with oxygenated TC medium 199 and then sealed with a second artery clamp. Using braided silk sutures, the tissue was divided into sacs (1.5 cm) either omitting or including PP tissue. Each sac was separated and pre-incubated for 5 min in Erlenmeyer flasks (50 ml) containing 9.0 ml of oxygenated TC medium 199 (37°C). Substrate (1.0 ml equivalent of 500 μg of b-chain in either its free or polymer-bound form, of which 50 μg was radioiodinated substrate) was added, the flasks were re-gassed and stoppered with silicon bungs and then left to oscillate (70 strokes/min) at 37°C in a water bath. At various times up to 75 min the sacs were removed, washed with ice-cold NaCl (0.85% w/v) and blotted dry. The contained serosal fluid was drained into LP3 tubes containing 1 M HCl (10 μl), and the gut sac tissue was dissolved in 1 M NaOH (25 ml). Samples of the tissue digest and serosal fluid were assayed for radioactivity, and the tissue digest assayed for protein using the Lowry protein assay (Lowry et al., 1951) as modified by Peterson (1983). The level of radioactivity in the gut sac tissue and serosal fluid at each time point was expressed as radioactivity or peptide/mg tissue protein. In addition, the serosal fluid and incubation medium samples were centrifuged (1500 × g, 4°C). The supernatant obtained was freeze-dried and stored at −70°C pending analysis by RP HPLC to identify the nature of the radioactivity. RP HPLC analysis was carried out as described above, with a radioisotope detector replacing the variable UV detector.

The ability of the intestinal sacs to maintain a glucose concentration [inside] > [outside] was taken as evidence of integrity and tissue viability throughout the course of an experiment. The glucose concentration in the medium and the serosal fluid was measured using a glucose oxidase-based test kit (Boehringer Mannheim).

3. Results

3.1. Preparation and characterisation of HPMA copolymer-peptide conjugates

The aminolytic reaction involves conjugation of two multifunctional compounds (Fig. 1); both peptides contain two primary amine groups and the HPMA copolymer precursor contains several *p*-nitrophenol groups (4.58 mol%). Use of a slight mole excess of *p*-nitrophenol groups to primary amine groups (2:1) was sufficient to facilitate 100% incorporation of b-chain (Fig. 2a) and peptide VP2 (Fig. 2b) as indicated by size-exclusion HPLC analysis of the reaction mixture. Both conjugates contained approximately 25% peptide (w/w), confirmed by quantitative analysis of the product using UV-spectroscopy (280 nm). Size-exclusion HPLC analysis of the HPMA copolymer-peptide conjugates compared with the aminolysed HPMA copolymer precursor showed that crosslinking reactions had not occurred during conjugation (Fig. 2a, Fig. 2b).

3.2. Degradation of b-chain and HPMA copolymer-b chain by BBM and luminal peptidases

The BBM and luminal enzyme preparations used were standardised by measuring release of *p*-nitroaniline during incubation with benzoyl-proylphenylarginine-*p*-nitroanalide. Neither storage at −70°C nor incubation throughout the degradation experiments at 37°C had any effect on the measured levels of enzyme activity.

When b-chain and HPMA copolymer-b chain were incubated (up to 2.5 h) with BBM enzymes RP HPLC revealed a similar pattern of degradation, there being three detectable degradation products (Fig. 3). The rate of appearance of the product with a retention time of 7.5 min was used to measure the initial velocity of both reactions (1 unit being taken as equivalent to 1 mm peak height/min). As an example the relationship between the product peak height and incubation time for a range of concentrations of free b-chain is shown in Fig. 4, and the initial velocity for the reaction was calculated from the linear region of each curve. Comparison of the initial rates of degradation of b-chain and HPMA copolymer-b chain by BBM peptidases as a function of peptide concentration showed that b-chain degradation was reduced up to 80% on coupling to HPMA (Fig. 5)

Incubation of free b-chain or HPMA copolymer-b chain with luminal peptidases gave six degradation products (Fig. 6). The rate of production of the fragment with a retention time of 13.3 min was used to measure the initial velocity of both degradation reactions (1 unit being taken as equivalent to 1 mm peak height/min). Again it was found that the conjugated peptide was degraded at a slower rate; the comparison of the rates of degradation of b-chain and HPMA copolymer-b chain as a function of peptide concentration showed a reduction in up to 60% (Fig. 5b).

3.3. Degradation of peptide VP2 and HPMA copolymer-peptide VP2 by BBM and luminal peptidases

When using a concentration of BBM and luminal enzymes (150 μg protein/ml) equivalent to that used for the degradation studies on b-chain and over the same incubation period, degradation of peptide VP2 did not occur. Therefore, the concentration of BBM and luminal enzymes was increased by 20- and 44-fold respectively to facilitate degradation. RP HPLC analysis of incubation mixtures containing peptide VP2 or the HPMA-peptide VP2 conjugate showed that on exposure to BBM enzymes four products resulted with six on incubation with the luminal enzyme preparation. However, as all these products were themselves subsequently degraded, the disappearance of the peptide VP2 peak itself was used to measure the initial velocity of the reactions, with 1 unit equiv-

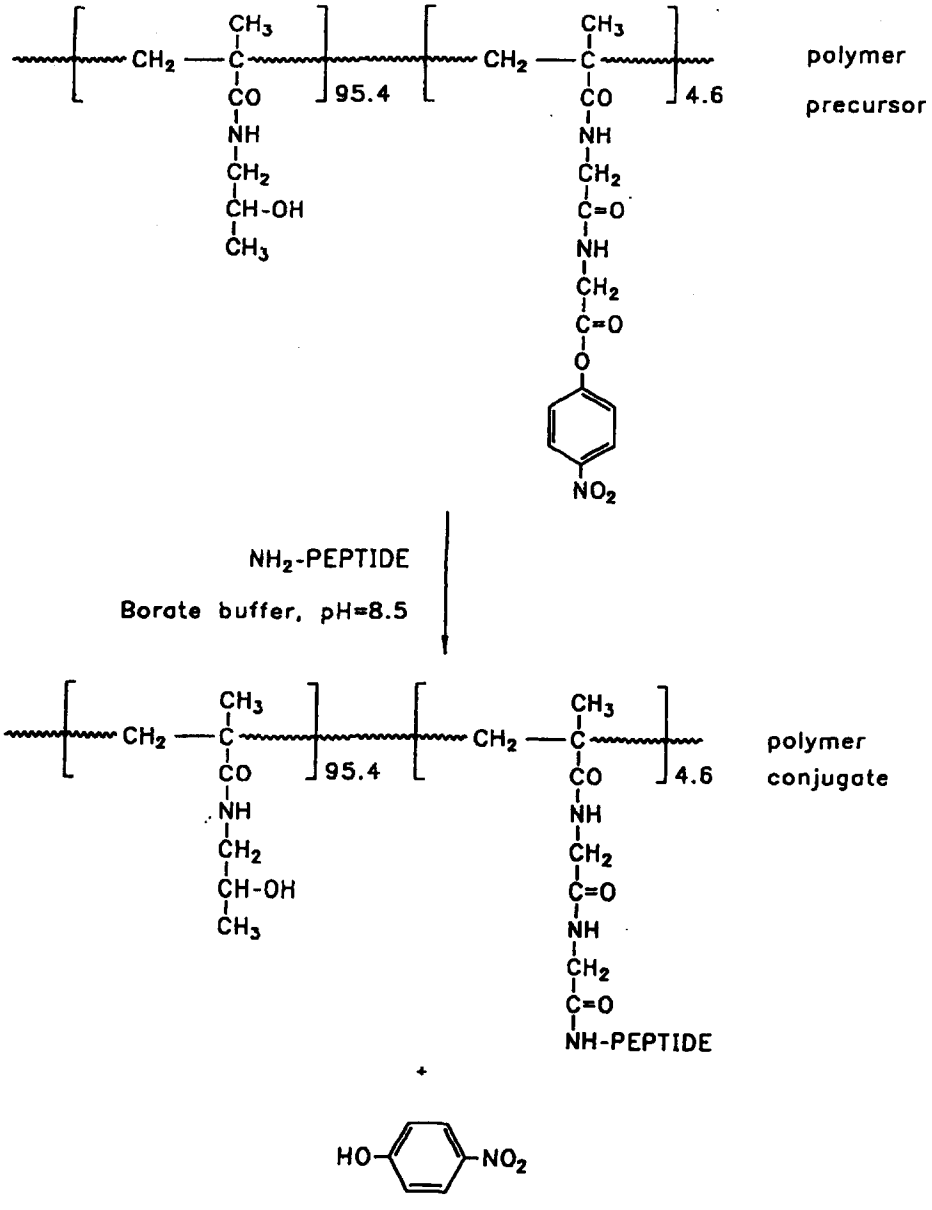

Fig. 1. Schematic of the conjugation pathway of HPMA conjugates. The aliphatic amino group of the peptide reacts with the *p*-nitrophenol ester of the HPMA precursor to form a covalent bond.

alent to 1 nmol peptide VP2 degraded/min (calculated from a calibration curve of known concentrations of peptide standards and respective peak heights). A comparison of the rates of degradation of peptide VP2 and HPMA copolymer-peptide VP2 by BBM peptidases as a function of peptide concentration showed that degradation was reduced up to a maximum of 80% on coupling to HPMA (Fig. 5c). The rates of degradation of peptide VP2 and HPMA copolymer-peptide VP2 by luminal enzymes are compared in Fig. 5d, and again it was found that

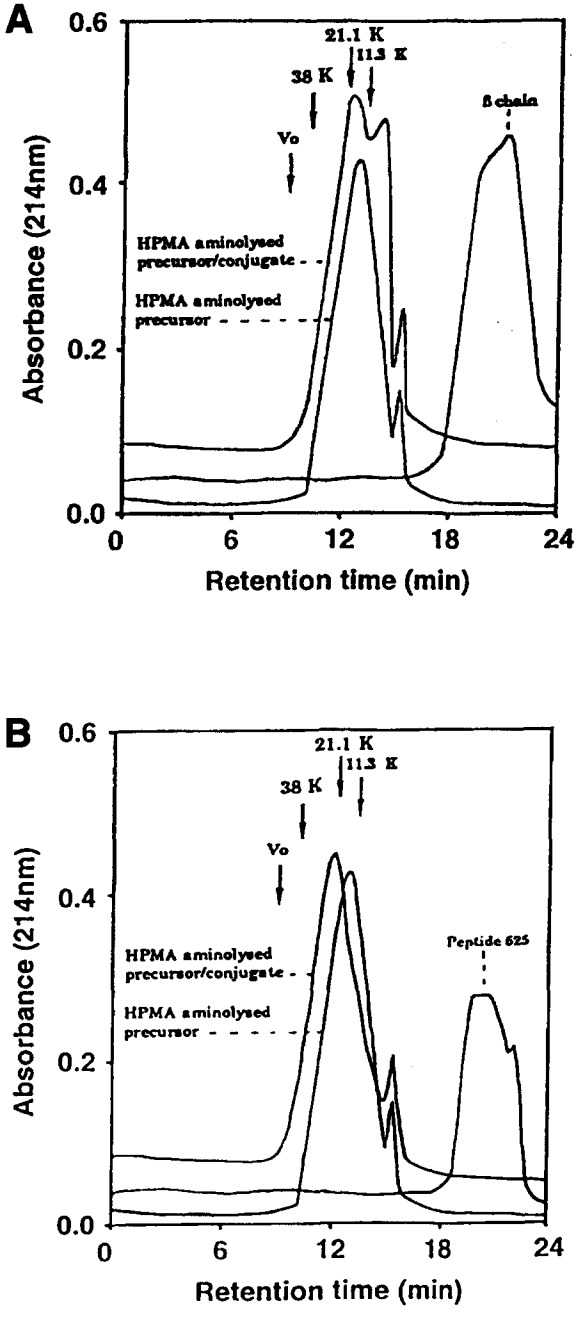

Fig. 2. Size-exclusion HPLC analysis of HPMA-peptide conjugates. The elution profiles of (a) HPMA aminolysed precursor, HPMA-b chain conjugate and free b-chain (b) HPMA aminolysed precursor, HPMA-peptide VP2 conjugate and free peptide VP2 are shown. Size-exclusion HPLC analysis was carried out as described in the methods.

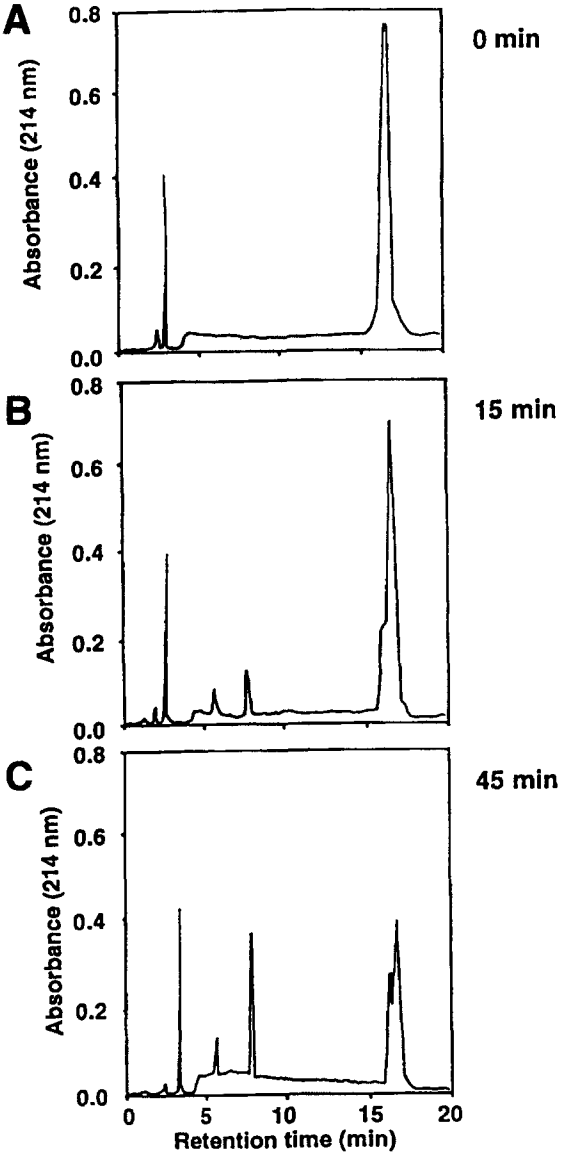

Fig. 3. Reverse phase HPLC analysis of b-chain degradation by rat small intestinal BBM peptidses. Analysis of samples after incubation for (a) 0 min, (b) 15 min and (c) 45 min. Reverse phase HPLC analysis was carried out as described in the methods.

conjugated peptide VP2 was degraded at a slower rate than free peptide with up to 55% reduction observed.

3.4. Uptake of ^{125}I-labelled b-chain and ^{125}I-labelled HPMA copolymer-b chain by adult rat everted gut sacs

The relative contributions made by enterocytes and M cells to the absorption of peptide was estimated by measuring transport of radioiodinated substrate across isolated rat jejunum using segments with or without PPs. Rat everted gut sacs were incubated with ^{125}I-labelled b-chain or ^{125}I-labelled HPMA copolymer-b chain, and the rate of transfer of ^{125}I-labelled material into the serosal fluid (contained within the gut sac) was measured (Fig. 7). A linear rate of transfer of radioactivity was observed in all cases. Following incubation with ^{125}I-labelled b-chain the radioactivity transported into the serosal fluid of sacs with or without PPs was equivalent to 576 and 377 ng peptide equivalent/mg tissue protein/h, respectively. In comparison, less radioactivity was transferred to the serosal fluid following incubation of sacs with ^{125}I-labelled HPMA copolymer-b chain; 400 and 198 ng peptide equivalent/mg tissue protein/h in sacs with or without PPs, respectively.

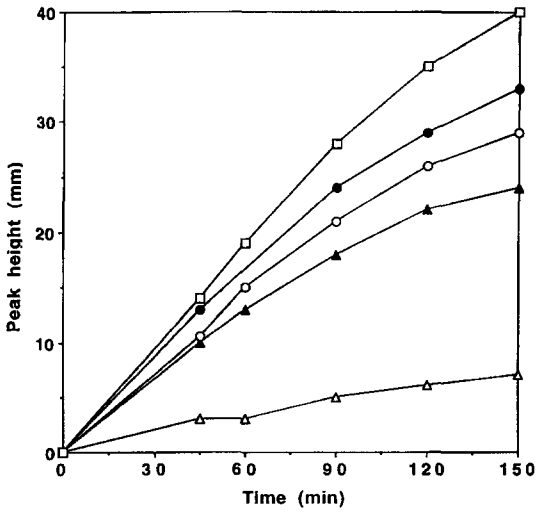

Fig. 4. The relationship between the concentration of b-chain (μM) and the rate of product formation on incubation with BBM enzymes; 154 (□——□), 123 (●——●), 100 (○——○), 80 (▲——▲) and 20 (△——△). Analysis was performed by reverse phase HPLC as described in the methods. The initial velocity of the reaction was calculated from measurement of peak height (mm) of one product with retention time 7.5 min (1 unit given as equivalent to 1 mm peak height/min).

The nature of the radioactivity recovered from serosal fluid was determined by RP HPLC (Fig. 8). Serosal fluid from gut sacs (both with and without PPs) incubated with ^{125}I-labelled b-chain corresponded only to degradation products, whereas serosal fluid from those sacs incubated with ^{125}I-labelled HPMA copolymer-b chain contained intact ^{125}I-labelled HPMA copolymer-b chain, with approximately 14% of the radioactivity recovered representing intact ^{125}I-labelled HPMA copolymer-b chain after a 75-min incubation. This corresponds to 66 and 58 ng of conjugated b-chain transported across per mg tissue protein in sacs with or without PPs, respectively.

There was no significant difference in the uptake of ^{125}I-labelled HPMA copolymer-b chain by sacs with or without PPs over the 75-min incubation period, but both showed a gradual plateau in transfer of the substrate in the serosal fluid after 30 min. This plateau could be explained by limited availability of substrate in the incubation medium due to peptide degradation. Indeed, on analysis by RP HPLC (data not shown), ^{125}I-labelled b-chain and ^{125}I-labelled HPMA copolymer-b chain were found to be stable in the incubation medium only (in the absence of gut sac tissue) for at least 3 h. However, 15 min after the addition of a gut sac (devoid of a PP), free ^{125}I-labelled b-chain was already completely degraded while 100% of the ^{125}I-labelled HPMA-b chain conjugate was still intact, although the ^{125}I-labelled conjugate was gradually degraded over a 60-min incubation period in the presence of the gut sac.

4. Discussion

An ideal polymer-peptide vaccine would have a high peptide loading, be free of cross-linking and intramolecular cycles, and also be free of unconjugated peptide. During synthesis of HPMA copolymer-insulin conjugates (Chytry et al., 1978) the Mw and degree of cross-linking was controlled by varying the ratio of primary amine to *p*-nitrophenol ester groups and the reaction time. This ap-

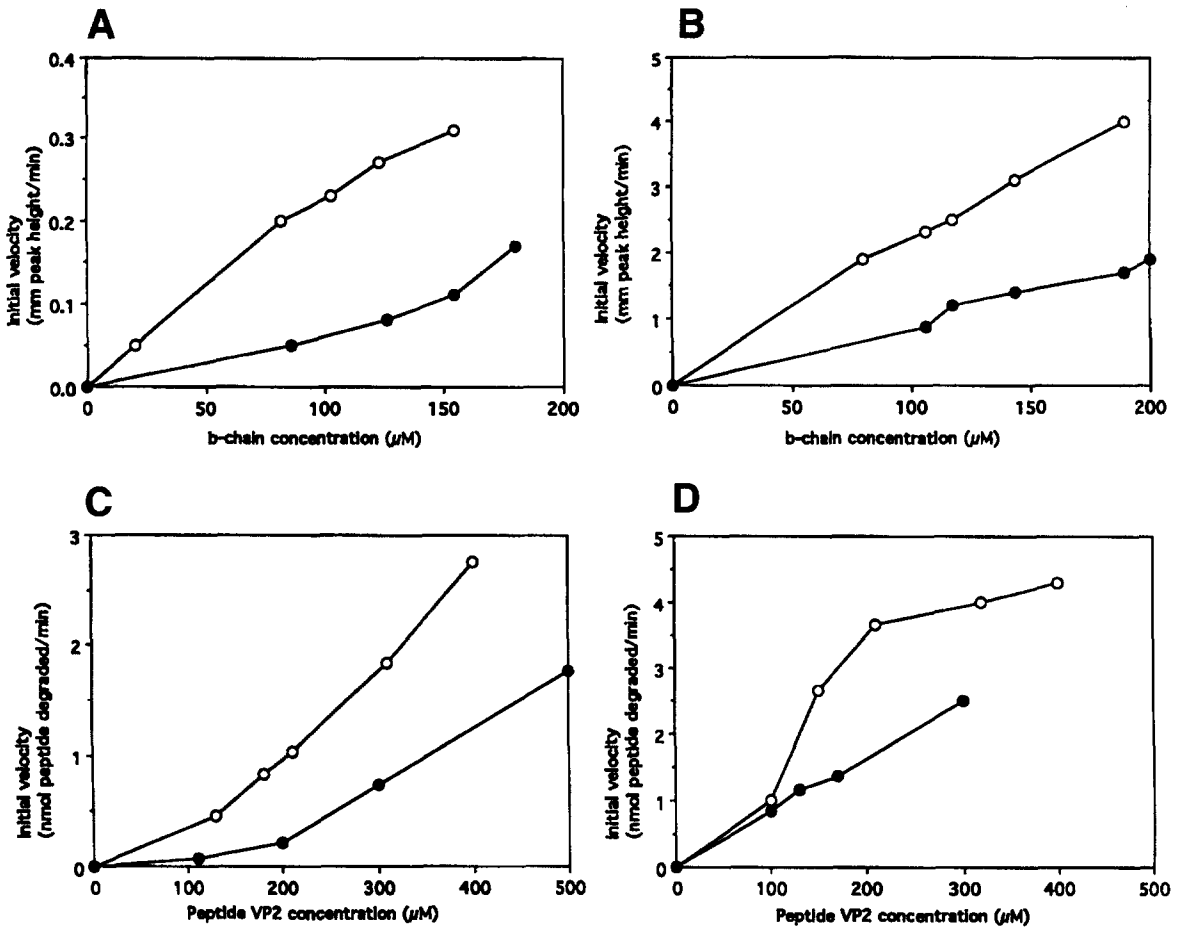

Fig. 5. The relationship between the concentration of b-chain (μM) and the rate of product formation on incubation with BBM enzymes; 154 (□——□), 123 (●——●), 100 (○——○), 80 (▲——▲) and 20 (△——△). Analysis was performed by reverse phase HPLC as described in the methods. The initial velocity of the reaction was calculated from measurement of peak height (mm) of one product with retention time 7.5 min (1 unit given as equivalent to 1 mm peak height/min).

proach proved unsuitable for synthesis of HPMA copolymer conjugates of b-chain and peptide VP2 as free peptide was always present in the reaction mixture at the end of conjugation (data not shown). Therefore, to avoid the purification step, an excess of polymer reactive groups (*p*-nitrophenol ester groups) to primary amine groups was used during the reaction (Rihova and Kopecek, 1985), The resulting conjugates produced had a relatively high peptide content (25% w/w), and as indicated by size-exclusion HPLC (Fig. 2a, Fig. 2b), no free peptide was observed and there was no evidence of crosslinking.

Free b-chain and peptide VP2 were clearly subject to extensive degradation by the mixtures of luminal and BBM enzymes. The Michaelis-Menten constant (Km) for the degradation reactions, which could only be calculated for b-chain, showed that peptidases from the luminal contents had a higher affinity for this peptide (0.67 mM and 0.38 mM with BBM and luminal peptidases, respectively). However, these kinetic constants are only theoretical values, calculated on the assumption that one peptidase cleaved at a single substrate site to yield the marker product used to assess the rate of degradation. In general it is

agreed that BBM peptidases preferentially hydrolyse peptides of up to 10 amino acid residues (Kania and Santiago, 1977; Matthews and Payne, 1980) and luminal peptidases have greater specificity for larger peptides (Nixon and Mawer, 1970a; Nixon and Mawer, 1970b). Peptide VP2 was found to be inherently more resistant than b-chain to degradation by both BBM and luminal peptidases, possibly due to specific conformational protection. It has been reported that whereas the T cell epitope of peptide VP2 maintained a ran-

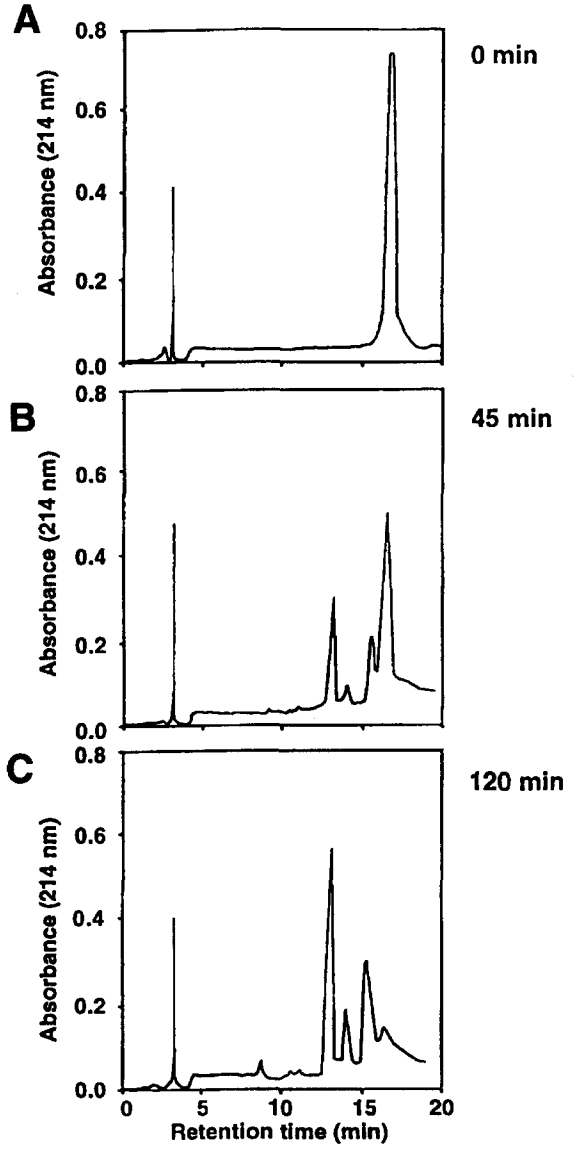

Fig. 6. Reverse phase HPLC analysis of b-chain degradation by rat small intestinal luminal peptides. Analysis of samples after incubation for (a) 0 min, (b) 45 min and (c) 120 min. Reverse phase HPLC analysis was carried out as described in methods.

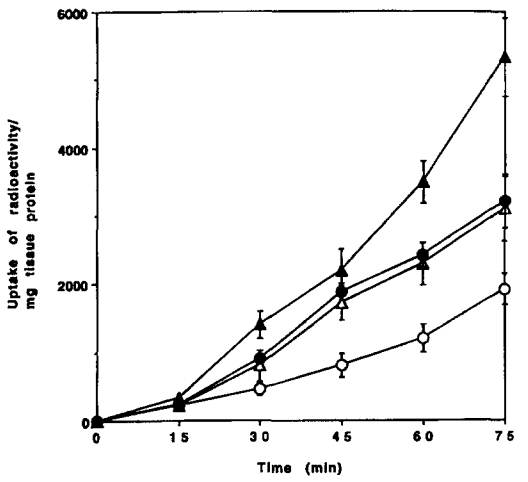

Fig. 7. Reverse phase HPLC analysis of b-chain degradation by rat small intestinal luminal peptides. Analysis of samples after incubation for (a) 0 min, (b) 45 min and (c) 120 min. Reverse phase HPLC analysis was carried out as described in methods.

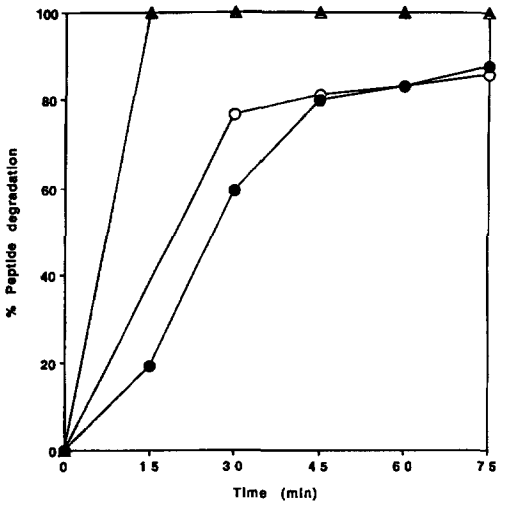

Fig. 8. Reverse phase HPLC analysis of b-chain degradation by rat small intestinal luminal peptides. Analysis of samples after incubation for (a) 0 min, (b) 45 min and (c) 120 min. Reverse phase HPLC analysis was carried out as described in methods.

dom coil in solution, the B cell epitope adopted an alpha-helical conformation (R. Campbell, unpublished data). Binding of b-chain and peptide VP2 to HPMA copolymer resulted in a marked reduction in degradation by both BBM (up to 80% for both b-chain and peptide VP2) and luminal peptidases (60% for b-chain and 55% for peptide VP2) over a wide range of substrate concentrations. Protection is probably a consequence of steric hindrance by the polymer backbone which interferes with formation of the enzyme-substrate complexes.

Data reported here confirm previous observations (Bridges et al., 1978; Bridges, 1980; Rowland and Woodley, 1981; Cartlidge et al., 1986) which show that small amounts of HPMA copolymer are absorbed by the adult rat small intestinal tissue. Here it was estimated that an average of 62 ng of conjugated b-chain was transported into serosal fluid/mg tissue protein. This represents an uptake of 248 ng of the HPMA-b chain conjugate/mg tissue protein. Peptides arriving in the small intestine can potentially be degraded by luminal and BBM peptidases or following internalisation intracellularly by lysosomal peptidases. Indeed, RP HPLC established that free b-chain was not transported into the serosal fluid, whereas an average serosal uptake of conjugate equivalent to 62 ng peptide/mg tissue protein/h, was observed both in everted gut sacs with or without PPs.

As we found no significant difference in the rate of capture of HPMA copolymer conjugates by tissue with or without PPs, conjugate modification to permit selective targeting of antigens to M cells might help to promote an immune response. Antigen-presenting cells from both the small intestinal PPs and diffuse lymphoid tissue of its lamina propria have been shown to present soluble protein antigen to naive T cells in vitro (Williams et al., 1990a; Williams et al., 1990b). Cultures of PP cells acting as antigen-presenting cells were found to consist primarily of Th cells, whereas cells isolated from the diffuse lamina propria were largely T suppressor cells. Selective targeting of peptide-based vaccines to M cells would be required to stimulate a protective mucosal immune response through activation of the appropriate Th cell response in the underlying PP tissue following transcytosis. There are a number of naturally occurring systems that could be used to facilitate targeting. For example, reovirus adheres to a receptor on M cells via a specific ligand, sigma 1 protein (Wolf et al., 1983). Linking this M cell adhesion protein to a HPMA copolymer-conjugate containing a peptide antigen would target the surface of M cells. Alternatively, attachment of wheatgerm agglutinin would also promote conjugate uptake by M cells (Owen and Bhalla, 1982).

The ability of HPMA copolymers to afford protection against degradation by small intestinal peptidases and to enhance absorption by the small intestinal tissue of covalently coupled peptides highlights an alternative approach to the particulate drug delivery systems which have recently received attention with respect to oral vaccine delivery. Eldridge and colleagues (Eldridge et al., 1989, 1990) reported that delivery of the staphylococcal enterotoxin B vaccine in microspheres < 5 μm or > 5 μm in diameter induced both antigen specific circulatory IgG and IgM, and disseminated mucosal secretory IgA responses, respectively. Even though such particulate formulations have achieved some success, the solvent reaction conditions necessary for their preparation can result in denaturation and ultimate destruction of the peptide or protein vaccine itself (J.R. Hanselaer, personnel communication). In comparison, the preparation of HPMA-peptide conjugate vaccines is carried out under mild aqueous reaction conditions. In addition, the HPMA copolymer backbone is chemically versatile, with the possibility of developing quite a sophisticated delivery system having the vaccine covalently coupled together with a targeting residue of choice (Duncan, 1992). To assess which type of drug delivery system is more suitable for in vivo administration, it would be necessary to compare both systems in parallel using the same model peptide vaccine. In vivo studies are underway to investigate the oral efficacy of the HPMA copolymer as a delivery system for peptide vaccines.

Acknowledgements

This work was supported by The Wellcome Foundation Ltd. (Beckenham, UK) and the Grant Agency of the Academy of Sciences of the Czech Republic. We thank R. Campbell (Beckenham, UK) for providing data on the conformation of peptide VP2 in solution.

References

Bridges, J.F., Uptake of Macromolecules by Rat Small Intestine In Vitro. Ph.D. Thesis, Keele University, UK, 1980.

Bridges, J.F., Millard, P.C. and Woodley, J.F., The uptake of liposome-entrapped ^{125}I-labelled poly(vinylpyrrolidone) by rat jejunum in vitro. *Biochim. Biophys. Acta*, 544 (1978) 448–451.

Britton, J.R., A method for the estimation of gastrointestinal luminal proteolysis of biologically important dietary peptides. *Nutr. Res.*, 9 (1989) 565–573.

Cartlidge, S.A., Duncan, R. and Lloyd, J.B., Soluble, crosslinked *N*-(2-hydroxypropyl)methacrylamide copolymers as potential drug carriers. (1) Pinocytosis by rat visceral yolk sacs and rat intestine cultured in vitro. Effect of molecular weight on uptake and intracellular degradation. *J. Control. Release*, 3 (1986) 55–66.

Chytry, V., Vrana, A. and Kopecek, J., Synthesis and activity of a polymer which contains insulin covalently bound on a copolymer of *N*-(2- hydroxypropyl)methacrylamide and *N*-methacryloyldiglycyl-*p*-nitrophenyl ester. *Makromol. Chem.*, 179 (1978) 329–336.

Crestfield, A.M., Moore, S. and Stein, W.H., The preparation and enzymatic hydrolysis of reduced and S-carboxymethylated proteins. *J. Biol. Chem.*, 238 (1963) 622–627.

Duncan, R., Drug-polymer conjugates: potential for improved chemotherapy. *Anti-Cancer Drugs*, 3 (1992) 175–210.

Eldridge, J.H., Meulbroek, J.A., Staas, J.K., Tice, T.R. and Gilley, R.M., Vaccine-containing biodegradable microspheres specifically enter the gut-associated lymphoid tissue following oral administration and induce a disseminated mucosal immune response. *Adv. Exp. Med. Biol.*, 251 (1989) 191–202.

Eldridge, J.H., Hammond, C.J., Meulbroek, J.A., Staas, J.K., Gilley, R.M. and Tice, T.R., Controlled release in the gut-associated lymphoid tissues. 1. Orally administered biodegradable microspheres target the Peyers Patches. *J. Control. Release*, 11 (1990) 205–214.

Francis, M.J., Hastings, G.Z., Campbell, R.O., Rowlands, D.J., Brown, F. and Peat, N., T-cell help for B-cell antibody production to rhinovirus peptides. In: Lerner, R.A., Ginsberg, H., Chanock, R.M. and Brown, B. (Eds.), *Vaccines* 89, Cold Spring Harbor Laboratories, New York, 1989, pp. 437–444.

Genco, R.J., Linzer, R. and Evans, R.T., Effect of adjuvants on orally administered antigens. *Ann. New York Acad. Sci.*, 409 (1983) 650–667.

Kania, R.J. and Santiago, N.A., Intestinal surface amino-peptidases. II. Substrate kinetics and topography of the active site. *J. Biol. Chem.*, 252 (1977) 4929–4934.

Kessler, M., Acuto, O., Storelli, C., Murer, H., Muller, M. and Semenza, G., A modified procedure for the rapid preparation of efficiently transporting vesicles from small intestinal brush border membranes. Their use in investigating some properties of D-glucose and choline transport systems. *Biochem. Biophys. Acta*, 506 (1978) 136–154.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, 193 (1951) 265–275.

Maloy, K.J., Donachie, A.M., O'Hagan, D.T. and Mowat, A.M., Induction of mucosal and systemic immune responses by immunisation with ovalbumin entrapped in poly(lactide-co-glycolide) microparticles. *Immunology*, 81 (1994) 661–667.

Marshall, J.S., Bienenstock, J., Perdue, M.H., Stanisz, A.M., Stead, R.H. and Ernst, P.B., Novel cellular interactions and network involving the intestinal immune system and its microenvironment. *APMIS*, 97 (1989) 383–394.

Matthews, D.M. and Payne, J.M., Transmembrane transport of small peptides. *Curr. Top. Membr. Transplant.*, 14 (1980) 331–425.

McFarlane, A.S., Efficient trace-labelling of proteins with iodine. *Nature*, 182 (1958) 53.

McGee, J.P., Davis, S.S. and O'Hagan, D.T., The immunogenicity of model protein entrapped poly(lactide-co-glycolide) microparticles prepared by a novel phase separation technique. *J. Control. Release*, 31 (1994) 55–60.

Merrifield, R.B., Solid phase peptide synthesis. 1. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.*, 85 (1963) 2149–2154.

Nixon, S.E. and Mawer, G.E., The digestion and absorption of protein in man. 1. The site of absorption. *Br. J. Nutr.*, 24 (1970a) 227–240.

Nixon, S.E. and Mawer, G.E., The digestion and absorption of protein in man. 2. The form in which digested protein is absorbed. *Br. J. Nutr.*, 24 (1970b) 241–258.

O'Hagan, D.T., Palin, K.J. and Davis, S.S., Poly(butyl-2-cyanoacrylate) particles as adjuvants for oral immunization. *Vaccine*, 7 (1989) 213–216.

Owen, R.L. and Bhalla, D.K., Cytochemical characterisation of enzyme distribution over M cell surfaces in rat Peyer's Patches. *Gastroenterology*, 82 (1982) 1144.

Peterson, G.L., Determination of total protein. *Methods Enzymol.*, 91 (1983) 95–119.

Rihova, B. and Kopecek, J., Biological evaluation of targetable poly-[*N*-(2-hydroxypropyl)methacrylamide]-antibody conjugates. *J. Control. Release*, 2 (1985) 289–310.

Rowland, R.N. and Woodley, J.F., Uptake of free and liposome-entrapped ^{125}I-labelled PVP by rat intestinal sacs in vitro: evidence for endocytosis. *Biosci. Rep.*, 1 (1981) 399–406.

Seymour, L.W., Flanagan, P.A., Al-Shamkhani, A., Subr, V., Ulbrich, K., Cassidy, J. and Duncan, R., Synthetic polymers conjugated to monoclonal antibodies: vehicles for tumour-targeted drug delivery. *Select. Cancer Ther.*, 7 (1991) 59–73.

Strohalm, J. and Kopecek, J., Poly *N*-(2-hydroxypropyl)-methacrylamide. I. Radical polymerization. *Angew. Makromol. Chem.*, (1978) 109–118.

Williams, N.A., Hill, T.J. and Hooper, D.C., Murine epidermal antigen presenting cells in primary and secondary T cell proliferative responses to herpes simplex virus in vitro. *Immunology*, 72 (1990a) 34–39.

Williams, N.A., Hill, T.J. and Hooper, D.C., Murine epidermal antigen presenting cells in primary and secondary T cell proliferative responses to a soluble antigen in vitro. *Immunology*, 71 (1990b) 411–416.

Wolf, J.L., Kauffman, R.S., Finberg, R., Dambrauskas, R., Fields, B.N. and Trier, J.S., Determinants of reovirus interaction with the intestinal M cells and absorptive cells of murine intestine. *Gastroenterology*, 85 (1983) 291–300.

Woodley, J.F., Peptidase activity in the GI tract: distribution between luminal contents and mucosal tissue. *Proc. Int. Symp. Control. Release Bioact. Mater.*, 18 (1991) 337–338.