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Body Distribution of Poly-*DL*-lactide–poly(ethylene glycol) Microspheres with Entrapped *Leptospira interrogans* Antigens Following Intravenous and Oral Administration to Guinea-pigs

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Abstract

Poly-*DL*-lactide–poly(ethylene glycol) (PELA) microspheres with entrapped antigens were administered intravenously and orally into guinea-pigs to quantitatively determine the in-vivo distribution and release profiles.

PELA microspheres containing ^{125}I-labelled outer-membrane protein *Leptospira interrogans* antigens (^{125}I-OMP) were prepared by double-emulsion solvent extraction procedure, and characterized with respect to size, morphology and in-vitro release profiles. The fractured sections of liver and spleen were inspected by scanning electron microscopy, which indicated that microspheres had successfully been entrapped within the above tissues after intravenous injection and oral administration. At predetermined intervals, the blood and such tissues as the liver, spleen, kidney, thyroid, small intestine and mesentery were collected, and the radioactivity was measured by gamma scintillation counting. Following intravenous administration, 56·7% of administered microspheres were accumulated in immunization-related tissues, and 40·1% of microspheres were located in the liver and spleen. However, there was limited uptake efficiency (8·33%) following oral administration, and 49·5% of the absorbed microspheres were located in the intestinal mucosa. Compared with in-vitro release, the in-vivo release profiles of ^{125}I-OMP from PELA microspheres, determined from the decreasing radioactivity in the above tissues, were much faster and the burst effect was higher.

Antigen-loaded PELA microspheres were efficiently entrapped within immunization-related tissues after intravenous administration, but orally administered PELA microspheres showed limited uptake efficiency. Further investigation is needed to improve intestinal absorption.

Immunization is the most important preventive action for protection against disease, disability and death from infection. The conventional multiple injection schedule often leads to dropouts among subjects to be immunized, causing failure of protection. Controlled release of vaccine antigens from biodegradable polymer microspheres is a promising technology for reducing the need to receive repeated immunization, protecting antigens from acidic and enzymatic degradation and providing either a continuous or a pulsatile antigen-release pattern (Cleland et al 1996; Singh et al 1997). In particular, lactide and glycolide copolymers (PLGAs) and poly-lactide (PLA) have been extensively investigated as matrices for releasing antigens. But PLGA and PLA show some drawbacks, resulting from their hydrophobic nature. The second component poly(ethylene glycol) (PEG), which has been widely used to improve the biocompatibility of blood-contacting materials, is introduced to form ABA block copolymer poly-*DL*-lactide-polyethylene glycol) (PELA) (Deng et al 1990). Compared with PLGA, PELA microspheres could promote the stability of proteins, increase the protein encapsulation efficiency in microspheres and

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decrease the burst-release of protein from microspheres, thus yielding a continuous release profile in-vitro (Deng et al 1999a; Li et al 1999a). In previous reports, the preparation of PELA microspheres with entrapped human serum albumin, outer membrane proteins of *Vibrio cholera* and *Leptospira interrogans*, were studied by investigating factors influencing the particle size and morphology, antigen encapsulation efficiency and in-vitro release profiles (Deng et al 1999b; Li et al 1999b).

Most of the vaccine studies reported to date involving controlled-release microparticles have concerned the optimization of preparative conditions and evaluation of immunogenicity. For the successful delivery of drugs or vaccines in particulate carriers, it is crucial that the quantitative extent of uptake is defined in the target organs. In many therapeutic situations, it is necessary to target other sites, and the uptake of particles by the liver and spleen can be a serious problem that must be overcome. In contrast, the efficient uptake of particles by macrophages in immunization-related tissues, such as the liver, spleen and intestinal Peyer's patches, makes an attractive way that antigen loading microspheres are administered parenterally or orally. Most studies have employed polystyrene microspheres with a diameter of 1–5 μm as a model particulate carrier, with adsorbed or entrapped fluorescent substance to form fluorescent microspheres (Eldridge et al 1990), to determine the in-vivo distribution of microparticles, and several research groups obtained different, even controversial, results (Damge et al 1996). PELA microspheres with hydrophilic PEG domains show different surface behaviours, believed to be attributable to different blood circulation levels and phagocytosis activities (O'Hagan 1996). Here we investigate the biodistribution in such tissues as liver, spleen and intestinal mucosa, and also the in-vivo release profiles of PELA microspheres after incorporation of ^{125}I-labelled outer-membrane protein (^{125}I-OMP) following intravenous and oral administration.

Materials and Methods

Materials

Copolymer PELA was prepared by ring-opening polymerization of DL-lactide and PEG using stannous chloride as initiator (Deng et al 1990). The weight-average molecular weight (MW) was 58·9 kDa with a polydispersity of 2·70, determined by gel-permeation chromatography (GPC, Waters ALC/GPC244). The PEG content was 10·5%, measured by ^{1}H NMR (Varian FT-80A). *Lepto-spira interrogans* antigen, outer membrane protein (OMP; MW 39 kDa) was obtained from the West China University of Medical Sciences. All other chemicals and solvents were of reagents grade or better.

OMP radiolabelling

OMP was labeled with ^{125}I by the chloramine-T method (Greenwood 1963). In brief, 10 μL Na^{125}I (1 mCi, Chinese Academy of Atom Energy, Beijing, China) and 20 μL of chloramine-T (5·4 mg mL^{-1}) was added to 40 μg OMP in 20 μL of 0·25 M phosphate buffer solution (PBS), pH 7·4. After stirring for 45 s in an ice-bath, the reaction was stopped by the addition of 250 μL sodium metabisulphite (4·8 mg mL^{-1}) and 100 μL 1% KI. Labeled OMP was purified over a Sephadex G25 column 1 × 14 cm after dilution with 5% barine serum albumin (BSA). The purified solution was diluted in PBS to a final volume of 5 mL. After the addition of 50 mg unlabelled OMP, the radioactivity of labelled antigen solution was measured by a gamma counter.

Preparation of OMP-PELA microspheres

OMP-loaded PELA microspheres were prepared by a previously reported w/o/w-solvent extraction procedure (Li et al 1999b). Briefly, an aqueous antigen solution was added to PELA dissolved in methylene chloride. The mixture was emulsified with a magnetic stirrer for 2 min to form the primary inner w/o emulsion. This emulsion was then added to the external water phase containing stabilizers and was emulsified again by a high-speed homogenizer. The organic solvent was extracted by adding 100 mL of 6% isopropanol and the mixture was stirred at a moderate speed at ambient temperature for 5–6 h. The antigen-loaded microspheres were centrifuged at 5000 *g* for 8 min and washed with double distilled water, and the procedure was repeated until the radioactivity in the supernatant water had reached a constant low level. The microspheres were then lyophilized overnight and stored at 4°C.

In-vitro characterization of OMP-PELA microspheres

A scanning electron microscope (SEM, Amray) was utilized to observe the topography and surface morphology of the OMP-PELA microspheres. The microspheres size was determined by a laser diffraction method using a particle size analyzer (Shimadzu SALD-2009, Japan), and expressed as

volume mean diameter. The level of residual dichloromethane within the microspheres was determined by gas chromatography (Shanghai Analytical Instrument Factory) with a set of standard concentrations of dichloromethane. The core loading of OMP in PELA microspheres was calculated by referring the percentage of radioactivity of encapsulated ^{125}I-labelled antigen to the total labelled OMP used to prepare microspheres.

In-vitro release of OMP was detected by incubating PELA microspheres into a test tube containing 154 mM PBS, pH 7·4. The tubes were kept in a thermostatted shaking water bath (Jiangsu Taichang Medical Apparatus Ltd, China) that was maintained at 37°C and 60 cycles min^{-1}. At predetermined intervals, three tubes for each sample were withdrawn, and 1·5 mL of the supernatant was collected by centrifugation, while the same volume of fresh PBS was added back to the tubes. The OMP content was determined from the radioactivity of the supernatant, and the mean of three values for each sample was calculated. As a control, the ^{125}I-labelled OMP solution was incubated under the same procedure to determine the natural decline of the radio isotope.

Animals

Guinea-pigs, ~250 g, were purchased from Chengdu Institute of Biological Products, Ministry of Public Health of China. Animals were kept under standard conditions, with free access to water and food.

Tissue distribution of PELA microspheres following intravenous and oral administration

PELA microspheres encapsulating ^{125}I-OMP were suspended in physical saline, corresponding to radioactivity of 20 μCi mL^{-1}. The guinea-pigs were divided into four groups. One group were anaesthetized and given a total volume per guinea-pig of 0·2 mL microspheres dispersion intravenously through the ear vein. The other group was given 1·0 mL of microspheres dispersion by gavage using a blunt tipped feeding needle inserted into the stomach. As a control, two groups were intravenously injected with 4 μCi ^{125}I-OMP solution or given 20 μCi of labelled antigen solution orally, respectively. At predetermined intervals (1, 2, 6 and 12 h and 1, 2, 3 and 6 days for the injected group; 6 and 12 h and 1, 2, 3 and 6 days for the peroral group), the guinea-pigs were killed and blood samples (about 1 mL) were taken. The guinea-pigs were dissected, and their liver, spleen, small intestine, thyroid, kidney and mesentery were removed and accurately weighed into test tubes. The blood and tissues' associated activities were counted using a gamma counter. A total blood volume per animal of 7·0% of body was assumed to determine the radioactivity of blood. At each time-point, three guinea-pigs for each group were measured, and results were expressed as the mean of three animals. The tissues were frozen in liquid nitrogen and cut into series sections, which were inspected by SEM to determine the targeting distribution of microspheres in guinea-pigs.

Statistical analysis

An unpaired Student's *t*-test was used to compare the radioactivity of the investigated tissues and to assess the statistical significance. $P < 0·05$ was considered significant.

Results and Discussion

Characterization of microspheres

The obtained OMP-PELA microspheres were presented as free-flowing powder, which was easily suspended in physical saline for administration. The SEM spectrum of antigen-loaded PELA microspheres displayed a smooth, spherical surface structure, with no evidence of collapsing. The obtained microspheres had a size range of 0·5–5·0 μm (the volume mean diameter: 2·68 μm, standard deviation: 0·169), and more than 96% of the population had a diameter of less than 5 μm. This is smaller than the diameter of capillaries. The residual dichloromethane present in microspheres was below 100 ppm measured by gas chromatography, which is below the limit according to the USP XXIII requirements (i.e., 500 ppm for methylene chloride) (Bitz & Doelker 1996). By comparing the radioactivity of microspheres dispersion with that of labelled OMP solution before encapsulation, an OMP loading efficiency of 74·7% into PELA microspheres was observed.

In-vitro release profiles

Figure 1 shows the percent release of OMP from PELA microspheres against incubation time. The release pattern was characterized as an at least biphasic process (i.e., an initial burst release followed by a gradual release phases). During the first day of the study, 16·1% of burst release was observed. The burst release mainly involves surface protein that has been partially or incompletely entrapped. In our PELA microspheres delivery

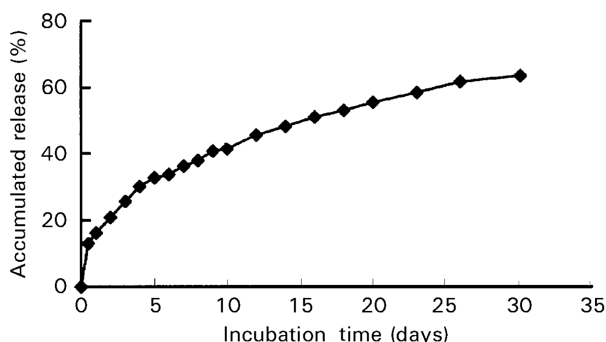

Figure 1. The in-vitro release profile of OMP from PELA microspheres. Each point represents the mean of three individual samples of microspheres.

system, the burst effect was lower by far than the 80% of burst release of ovalbumin from PLA microspheres observed by Uchida et al (1994). This may be because of the preferential localization of protein molecules within the deeper sections of PELA microspheres matrix, due to the existence of hydrophilic PEG domains of PELA.

In this study, a steady release of OMP over 30 days was observed. In the in-vitro protein release from PLA or PLGA microspheres, an initial burst release following a lag phase (characterized as no protein release) was observed (Cohen et al 1991). It is suggested that the gradual release of protein from PELA microspheres is effected by the swollen inner structure and porous network formed by the microspheres' matrices contacting the aqueous release medium, protein diffusion initially and polymer degradation. As seen from Figure 1, 37% of OMP remained in the microspheres after the one-month test. This might be due to the low diffusion rate caused by low protein loading during the later incubation time, and the electrical interactions between the basic amino acids of the protein and the acidic carboxyl groups of the degraded polymer.

In-vivo behavior of PELA microspheres following intravenous injection

Following intramuscular or subcutaneous injection, microspheres of 0·5–5·0 μm size remain at the injection sites initially, and then migrate into the venous circulation. Our aim was to investigate the in-vivo behaviour of PELA microspheres following intravenous injection into guinea-pigs. Intravenously injected particulate substance or drug carriers with an average size below 7 μm are normally taken up by macrophages of the mononuclear phagocytic system (MPS), particularly by the Kupffer cells of the liver. This was also apparent for PELA microspheres. The targeting distribution of microspheres in such tissues as the intestinal peyer's patches, liver and spleen was qualitatively detected by SEM. Figure 2 shows the PELA microspheres locating in liver and spleen of animals two weeks after intravenous injection.

The quantitative distribution of PELA microspheres with entrapped ^{125}I-OMP in different organs was studied over a period of 6 days after intravenous administration. There was little inter-animal weight variation and the same dose of radioactivity was used in each animal, thus the radioactivity of whole tissues were determined to evaluate the in-vivo distribution of PELA microspheres. The data for thyroid (0·007 μCi g^{-1}) was found to be negligible, compared with those such as the liver (0·093 μCi g^{-1}), spleen (0·141 μCi g^{-1}) and mesentery (0·116 μCi g^{-1}), for example. The radioactivity in the blood, liver, spleen, kidney, mesentery and small intestine was determined and summarized in Table 1. As seen from Table 1, 53·9% of microspheres was accumulated into such tissues as liver, spleen, mesentery and intestinal mucosa 1 h after injection. Considering that 5% of

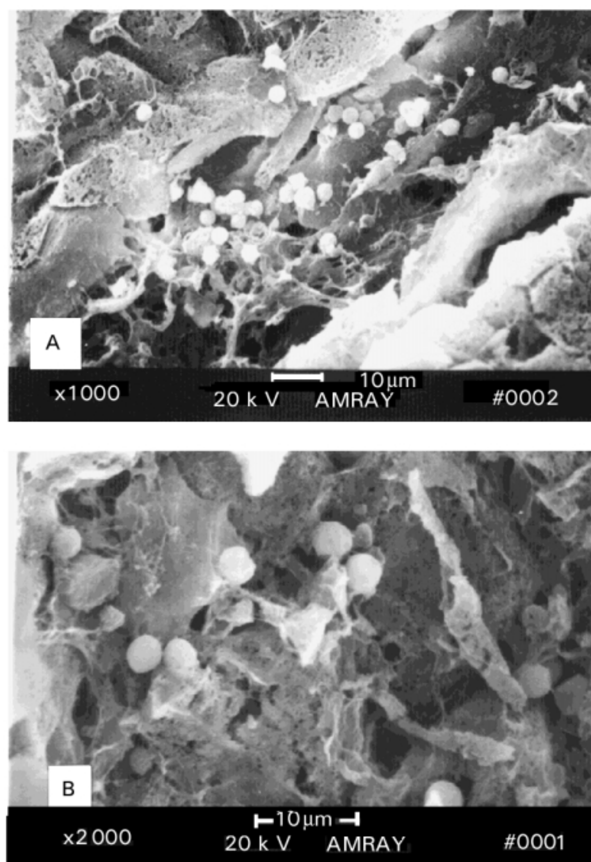

Figure 2. The SEM spectrum of fractured sections of liver (A) and spleen (B) of guinea-pigs two weeks after intravenous immunization with OMP-PELA microspheres.

Table 1. Organ distribution of ^{125}I-OMP-loaded PELA microspheres after intravenous injection to guinea-pigs.[a]

Time	Radioactivity after treatment (μCi)					
	Liver	Spleen	Mesentery	Small intestine	Blood	Kidney
1 h	1·050 ± 0·126	0·475 ± 0·033	0·285 ± 0·041	0·345 ± 0·056	0·217 ± 0·016	0·070 ± 0·006
2 h	0·769 ± 0·092	0·275 ± 0·015	0·107 ± 0·016	0·176 ± 0·027	0·124 ± 0·010	0·054 ± 0·006
6 h	0·591 ± 0·071	0·188 ± 0·014	0·082 ± 0·011	0·069 ± 0·013	0·097 ± 0·008	0·042 ± 0·003
12 h	0·470 ± 0·083	0·110 ± 0·009	0·076 ± 0·011	0·036 ± 0·009	0·056 ± 0·005	0·041 ± 0·004
1 Day	0·343 ± 0·047	0·076 ± 0·004	0·061 ± 0·008	0·043 ± 0·004	0·052 ± 0·003	0·038 ± 0·004
2 Days	0·211 ± 0·021	0·057 ± 0·004	0·052 ± 0·007	0·024 ± 0·004	0·045 ± 0·004	0·037 ± 0·005
3 Days	0·164 ± 0·019	0·039 ± 0·004	0·041 ± 0·007	0·028 ± 0·003	0·051 ± 0·002	0·033 ± 0·001
6 Days	0·160 ± 0·027	0·025 ± 0·006	0·039 ± 0·004	0·017 ± 0·004	0·053 ± 0·006	0·021 ± 0·003

[a]Dose, 4 μCi per animal. Values are means ± s.d.

OMP is released from the microspheres during the initial hour (Figure 1), 56·7% of microspheres have reached the above tissues. According to statistical analyses, there existed significant differences between the radioactivity of the liver and that of the other five tissues (blood, spleen, kidney, small intestine and mesentery) ($P < 0·05$), while no significant difference existed among these five tissues ($P > 0·05$). The liver and spleen are normally sites for the deposition of small particles, due to the existence of macrophages (e.g. Kupffer cells in livers that will engulf foreign particles). The major site for particle deposition was the liver and spleen (40·1% of dosed microspheres). Through the venous circulation, the microspheres reached the small intestine (8·6% of dosed microspheres 1 h post-administration), where large amounts of macrophages were presented.

The PELA microspheres entrapped within the above tissues would experience a gradual loss of ^{125}I-OMP. The released OMP would principally migrate from these tissues into the venous circulation, then accomplish immunization and (or) be eliminated into the urine. Figure 3 shows the total radioactivity in the liver, spleen, mesentery and small intestine after intravenous injection of OMP-loaded PELA microspheres and ^{125}I-OMP control solution. Levels of radioactivity were approximately 3 times higher when it was encapsulated into microspheres than after administration of the control solution 1 h post-administration. The difference between these formulations increased with increasing time. Twelve hours after injection, 19·7% of the dosed OMP was found in the above four tissues, whereas only 2·6% of the label was found after injection of the control solution. Thus, the data shown in Table 1 may be regarded approximately as an in-vivo release profile. The area under the curve of the microspheres was 9·2 times higher than that of the solution. Sustained release is achieved by the slow leaching of protein from microspheres entrapped in the above tissues, which would stimulate a significant immune response following the single-injection vaccination method.

The decrease in radioactivity of tissues shown in Figure 3 was due to OMP release from microspheres. A significant drop of radioactivity preceded a slow reduction, which was in agreement with the burst release of OMP during the initial days and a gradual release in the following incubation time, as observed in the in-vitro release investigation of OMP-loaded PELA microspheres (Figure 1). Comparing the release profiles shown in Figures 1 and 3, the burst effect of PELA microspheres resulting from in-vivo investigation was more noticeable than their in-vitro behaviour. The different release patterns in-vivo and in-vitro could

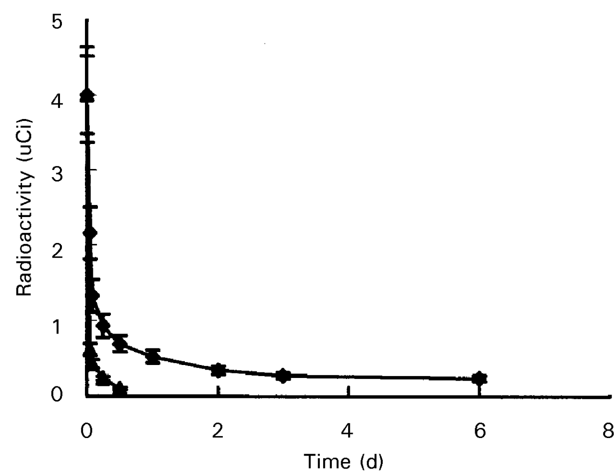

Figure 3. The total activity of liver, spleen, mesentery and small intestine vs time after intravenous injection of ^{125}I-OMP-loaded PELA microspheres dispersion (◆) and ^{125}I-labelled OMP solution (▲) to guinea-pigs.

be explained by different degradation profiles. It is difficult, however, to recover microspheres from body circulation and organs, either by intramuscular, intravenous or oral administration, and analyse their degradation behaviour. Studies are currently under way to try to examine in-vivo degradation and find another in-vitro release assay that better corresponds to in-vivo behaviour.

In-vivo behaviour of PELA microspheres after oral administration

The oral route is a relatively simple, convenient and safe method of vaccination. Consequently, the design of oral vaccine formulations may be a promising improvement in the immunization coverage and individual's compliance. Antigen molecules can be incorporated into biodegradable microspheres to reduce molecular breakdown in the gastrointestinal acidic and enzymatic environment. The possibility of uptake and absorption of microparticles by the gastrointestinal tract has been a controversial issue, although there is now accumulated evidence that it does occur. The uptake of microparticles through the gut wall is accepted as a true biological phenomenon. Thus, the absorption of bioactive agents in an unaltered form is enabled. Fractions of liver and spleen were inspected by SEM, which indicated that PELA microspheres had successfully reached the targeting sites of immunization-related tissues. Figure 4 shows the PELA microspheres were located in the liver and spleen of mice two weeks after oral administration.

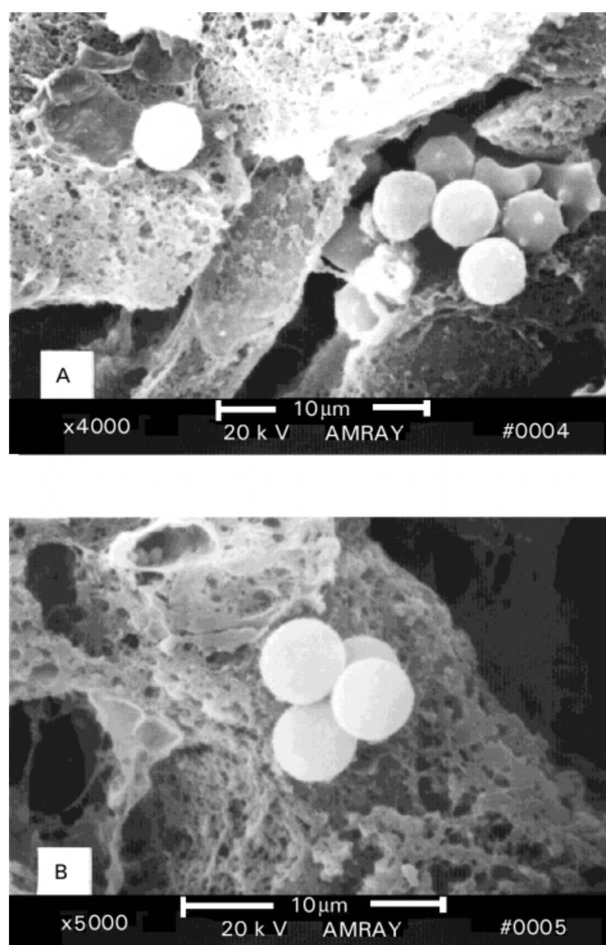

Figure 4. The SEM spectrum of fractured sections of liver (A) and spleen (B) of guinea-pig after oral immunization with OMP-PELA microspheres.

The extent of particle uptake remains poorly defined. There has been relatively little attention paid to quantitatively determining the extent of uptake. Most studies aimed at determining the extent and mechanisms of particulate translocation across gastrointestinal epithelia have employed polystyrene microspheres as a model. Concerning the extent of absorption of particles, the results reported in the literatures have been controversial. Alpar et al (1989) showed that 39% of 1·1-μm fluorescent polystyrene particle were absorbed in the rat after intragastric administration. Jani et al (1990), who administered 1·0-μm polystyrene particles to rats, found an absorption rate of 5%. In contrast, Ebel (1990) found a very low uptake of 2·65-μm polystyrene particles (lower than 0·01%). These different results reflect that it is difficult to define the efficiency of the passage of particles through the gastrointestinal tract and that the importance of uptake of particles by Peyer's patches remains uncertain. Moreover, results derived from such studies can not be extrapolated reliably to the present PELA microspheres, because of the difference in polymer characteristics and surface properties between PELA and polystyrene microspheres.

Due to the variation of transit time between animals (5–24 h in rates) (Damge et al 1996), it is difficult to evaluate correctly the kinetics of absorption of particles through the intestinal mucosa. Table 2 summarizes the radioactivity in the blood, liver, spleen, kidney, mesentery and small intestine. The total amount of microspheres absorbed at the intestinal mucosal site was approximately 7·5% 6 h post-administration, and 49·5% of the absorbed microspheres (3·72% of the total dose) were located in the small intestine. Considering that 10% of the OMP was released from the microspheres during the initial 6 h (determined from Figure 1), only 8·33% of microspheres were adsorbed at the intestine and then transported from the lymphatic circulation under the intestinal mucosa layer into the venous circulation, and subsequently into the liver and spleen. According to statistical analyses, the radioactivity

Table 2. Organ distribution of ^{125}I-OMP loaded PELA microspheres after oral administration to guinea pigs.[a]

Time	Radioactivity after treatment (μCi)					
	Liver	Spleen	Mesentery	Small intestine	Blood	Kidney
6 h	0·220 ± 0·024	0·040 ± 0·003	0·196 ± 0·029	0·743 ± 0·122	0·278 ± 0·027	0·023 ± 0·004
12 h	0·350 ± 0·031	0·080 ± 0·005	0·140 ± 0·021	0·615 ± 0·113	0·175 ± 0·016	0·060 ± 0·009
1 day	0·283 ± 0·031	0·040 ± 0·003	0·096 ± 0·014	0·368 ± 0·063	0·092 ± 0·011	0·042 ± 0·004
2 days	0·178 ± 0·016	0·020 ± 0·002	0·074 ± 0·012	0·227 ± 0·039	0·063 ± 0·006	0·028 ± 0·004
3 days	0·138 ± 0·017	0·018 ± 0·002	0·050 ± 0·009	0·163 ± 0·031	0·055 ± 0·007	0·016 ± 0·001
6 days	0·114 ± 0·015	0·014 ± 0·002	0·035 ± 0·005	0·087 ± 0·021	0·048 ± 0·004	0·014 ± 0·003

[a]Dose, 20 μCi per animal. Values are means ± s.d.

of the liver and small intestine were significantly higher than that of the blood, spleen, kidney and mesentery ($P < 0{\cdot}05$), while no significant difference existed between the radioactivity of the liver and the small intestine ($P > 0{\cdot}05$). Thus, the OMP-loaded PELA microspheres initially destined to the small intestine and liver after oral administration.

Although PELA microspheres lack efficient uptake, evident immune responses have been achieved by oral delivery of vaccines (Jia et al 1997). The saliva and faecal IgA titer in peroral PELA microspheres with entrapped *Vibrio cholera* antigens was 5 times higher than that of fluid antigen at 6 weeks post-administration, and 10 times higher at week 12. Immune preventive efficiency was 50–70% for animals vaccinated with peroral microspheres, but only 10% with fluid antigen solution for mice challenged with virulent *V. cholera* strain Inada 18003. This may be due to the fact that the predominant site of uptake, the small intestinal mucosa, is the inductive site for mucosal immunity. The majority of pathogens enter the host via a mucosal surface and the potential mucosal immunity may offer an effective first line of defence. Orally administered PELA microspheres show limited uptake efficiency, therefore investigations are in progress to develop means of increasing intestinal absorption.

Figure 5 shows the decrease in radioactivity of the small intestine, mesentery, liver and spleen of PELA microspheres and control solution. Due to the sustained release of OMP from microspheres, a significant drop of radioactivity preceded a slow reduction. For the OMP control solution, limited uptake at the intestinal mucosa was observed. The radioactivity was detected in the small intestine and mesentery (1·6% of OMP dose 6 h post-administration), and decreased significantly with time. The distribution in the liver and spleen through the venous circulation remains no detecting throughout the investigation period.

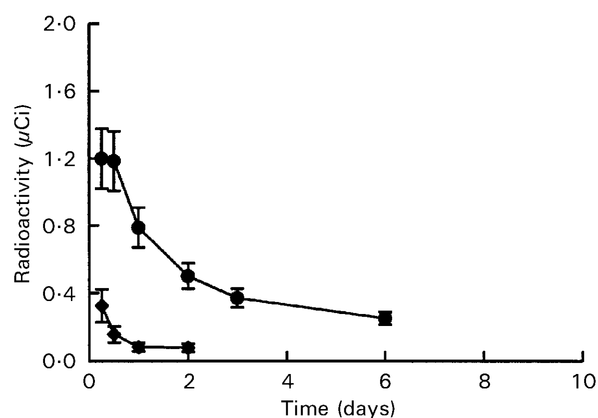

Figure 5. The total activity of liver, spleen, mesentery and small intestine vs time after oral administration of ^{125}I-OMP-loaded PELA microspheres dispersion (◆) and ^{125}I-labelled OMP solution (▲) to guinea-pigs.

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