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• *H.pylori* •

PELA microspheres loaded *H. pylori* lysates and their mucosal immune response

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Abstract

AIM: To prepare poly (D,L-lactide)-polyethylene glycol copolymer (PELA) microspheres loaded *H.pylori* lysates or *Cystografin* and observe their targeting in gastrointestinal mucous membrane or analyze the mucosal immune responses by oral administration.

METHODS: PELA microspheres loaded *H.pylori* lysates or *Cystografin* were prepared by double emulsion evaporation method. Their distribution in gastrointestinal mucous membrane was observed by CT.Balb/c mice orally immunized in mucosal immune responses, whose antibody production in salivary and gut washing and antibody secreting cells in Peyer's patches (PP) were estimated by ELISA and ELISPOT, respectively. The microspheres' physical properties, such as particle size, protein level and morphology were investigated.

RESULTS: All prepared microspheres were found to have a smooth surface morphology from 3.20-4.05 μm in diameter and high encapsulation efficiency from 74.9-82.2 %. No significant correlation in their physical properties was shown, depending on their molecular weight at the similar composition ratio. Immunization with all types of PELA-*Hp* microspheres elevated the saliva sIgA level at week 3 by approximately 3-4 times that with soluble antigen, which was greatly enhanced after boosting. At one week after last immunization with all types of PELA-*Hp* microspheres (week 8), the specific sIgA-ASCs, IgG-ASCs and sIgA in salivary rose obviously. In intestinal Peyer's patches, the specific sIgA-ASCs were 5.92-6.98$\times 10^4$/ml cell and IgG-ASCs were 3.47-4.02$\times 10^4$/ml cell, about 5-9 times higher than those with soluble antigen ($P<0.01$). ASCs in intestine were more than those in stomach and the majority of the ASCs were sIgA-ASCs. The sIgA in gut washing fluid was 1.62-1.85 OD, about 3-6 times tthat of those with soluble antigen. There were significant differences of the ASCs and sIgA in gut washing fluid as compared with those of PBS and MS-0 ($P<0.05$). There appeared to be good correlation between sIgA level in gut washing fluid and sIgA-ASCs in intestinal Peyer's patches.

CONCLUSION: PELA microspheres may be used as vehicle to delivery antigen and adjuvant in designing oral vaccination.

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INTRODUCTION

Helicobacter pylori (*Hp*) is a major pathogen causing type B gastritis, peptic ulcer and mucosa-associated lymphoid tissue gastric lymphoma[1-12]. A triple antimicrobial therapy is often adopted for patients with infection[13-16], for *Hp* reinfection easily occurs and the drug resistance strains are increasing, therefore, antimicrobial treatment may be ineffective in prevention of reinfection[17-21]. Oral immunization is considered a convenient and safe method to induce mucosal immunity against infection. This has become a focus topic among the researchers[22-26].

The biodegradable and biocompatible microsphere as a vaccine delivery vehicle has many advantages[26]. The biodegradable polyesters (including polylactic acid (PLA), polyglycolic acid (PGA) and their copolymers (PLGA)) had been widely studied and used in biomedical engineering[27-30]. For hydrophobicity of the PLA and the PLGA, this type of material is usually not desirable for protein and peptide. Their degraded products, lactic acids or glycolic acids will create a local acidic environment that may be harmful to the surrounding tissues[31]. These microspheres loaded vaccines can be rapidly captured by phagocyte in the reticulo-endothelial system, while the microspheres (nanospheres) prepared with PLA-PEG-PLA (type A-B-A) triblock copolymers, which was produced by copolymerization of hydrophilic polyethylene glycol with lactide, showed longer circulating half life of the proteins *in vivo*[32]. They can overcome the disadvantages of the PLA or PLGA microspheres as drug vehicle.

MATERIALS AND METHODS

Bacterial culture

*Hp*971023, *Hp*980706, *HpM* bacterial strains were isolated by our laboratory. The strains were inoculated onto blood plat in the microaerobic cultivation at 37 ℃ for 48 h. The organisms were washed 3 times with 0.15 mol/L phosphate buffered saline (PBS, pH7.4) and were harvested by centrifugation at 5 000rpm for 10 minutes at 4 ℃. The resulting suspensions was added to 0.15 mol/L PBS(pH7.4), EDTA 0.65 g/L and phenylmethylsufonyl fluoride (PMSF) 1 mmol/L and sonicated (200W$\times$30s$\times$10 times). The *Hp* lysate was collected by centrifugation at 12 000 rpm for 20 minutes. The protein concentration was determined by UV spectrophotometer (Beckman DU-640, USA).

Preparation of the PELA microspheres loaded Hp

PELA (weight ratio of D, L-lactide to PEG-2000, 95:5; the inherent viscosity of the PELA ranged from 0.1271 to 0.3329 dL/g measured in tetrahydrofuran at 25 ℃) was synthesized

according to procedures described in literature[32]. The PELA microspheres loaded *Hp* were manufactured using double emulsion evaporation method as described[32,33]. One ml aqueous solution mixed 4 % *Hp* lysate (inter-water phase) was emulsified with 10 ml of 6 % (w/v) PELA in methylene chloride using T25B homogenizer (USA) at 8 000 rpm for 5 min (w/o), After homogenization, 30 ml aqueous solution of 2 % PVA (degree of polymerization n = 1 500-1 800) was added to the primary water-in oil (w/o) emulsion and the stirring was continued further for 5 min. The resulting w/o/w suspensions were stirred magnetically at 1 200 rpm for 10-12 h at room temperature (over 25℃) to evaporate the solvent. The microspheres were obtained by centrifugation and washed three to eight times with distilled water. The cleaned microspheres were lyophilized and stored at 4 ℃ under desiccation. If the aqueous solution mixed *Hp* lysate (inter-water phase) was replaced by pure water or some concentration of *cystografin*, the blank PELA microspheres and PELA-*cystografin* microspheres can be obtained respectively. The amount of protein loaded in the PELA microspheres was determined by dissolving a fixed amount of microspheres in methylene chloride, the protein content was measured with UV spectrophotometer (Beckman DU-640, USA). The protein loading efficiency was calculated directly by recovering the protein from the microspheres[34].

Measurement of microsphere size and morphology

The mean size of microspheres and distribution were calculated using a LeitzDiaplan light microscope (Wild MPS52, Japan) to measure the diameter of microspheres, whose amount was no less than 200. The surface structure of micropheres was examined by scanning electron microscopy (AMRAY, USA-China).

PELA-cystografin microspheres targeting

Miniture pigs (*sus scorfa domestica*) weighing 15-20 kg were used in the study. PELA-*cystografin* microspheres (mean diameter of 3.72 μm, the amount of *cystografin* loaded was about 16 %) were suspended in 7.5 % sodium bicarbonate solution and 100 ml suspension (10 g PELA-*cystografin* microspheres) was orally administered by intubation feeding to miniture pigs. After 0, 3, 8 and 15 days, the distribution of PELA-*cystografin* microspheres in the all gastrointestinal tract was observed by CT. The pigs were killed and the images of all gastrointestinal tract were taken after being washed up.

Immunization procedures

BALB/c mice (female, 6-8 weeks old, and 18-25 g in weight) were offered by our experimental animal center. Mice were randomly assigned to one of the seven immunization groups (twenty mice per group). Before administration, mice stopped feeding for 2 h and then took orally 0.5 ml mixture of the Hanks' equilibration salt and 7.5 % sodium bicarbonate solution (4:1 ratio) in order to neutralize gastric acid fluids. Each experimental group was orally immunized on week 0, 1 and 2. The immunized mice were given a booster immunization on week 7 with the same agents as the primary immunization. The first four groups were immunized by oral administration with different PELA-*Hp* microspheres (that is MS-1, MS-2, MS-3 and MS-4 group, 1mg *Hp* per mouse). The fifth group, sixth and seventh group of mice received free 1 mg of souble *Hp* lysate antigen, PBS solution or blank PELA microspheres under similar conditions, respectively. One week after the last immunizations (week 8), mice were killed and assayed by indirect ELISA and ELISPOT.

Detection of ASCs in lymphocytes from intestine and gastric mucosa

Peyer' s patches (PP) in intestine and gastric mucosa were selected[35] and placed into 0.5 ml 5 % FCS RPMI-1640 medium. The tissues were ground and recovered on 70 μm cell strainer. The recovered tissues were harvested by centrifugation at 1 500rpm for 15 minutes and washed two times to collect suspensions, which were added 15 ml 70 % Percoll medium and centrifuged at 1500rpm for 15 minutes. Cell pellets are resuspended in 0.5 ml RPMI-1640 medium to form 5×10^5/ml. The cell suspensions were stained with 2 % trypan blue. The cell viability and purity were assessed. The ELISPOT assay[36] was used to detect ASCs in the intestine and gastric tissues of the mice. Briefly, 96-well (Millipore) were coated with 0.2 ml *H. pylori* sonicate preparations dissolved in 0.05 mol/L sodium bicarbonate solution (50 μg protein per well) and left overnight at 4 ℃. After washed three times in PBS, well plates were saturated with 2 % BSA (100 μl per well) and incubated at 37 ℃ for 1 hour. The plates were washed once in RPMI-1640 and each well was added 0.2 ml 5×10^5/ml of the cell suspensions.

The cells were incubated at 37 ℃ in 10 % CO_2 for 4 hours. The plates were washed three times in PBS-Tween and then added 100 μl biotinylated monoclonal IgA or IgG antibodies to specific mouse , which had been diluted 1:1500 and 1:2000 in 10 % FCS-PBS, respectively. The plates were incubated at 37 ℃ for 2 hours. After three washes, the avidinperoxidase solution, diluted 1:2 000 in PBS, was incubated at 37℃ for 30 minutes. After three washes in PBS-Tween, 100 μl substrate solution (containing 7.5 % gelatin, 1 g/L 3,3', 5,5'-tetramethylbenzidine (TMB), 0.03 % H_2O_2) was added to each well. The reaction was coagulated quickly in ice-water bath. After 10-30 minutes, the immunospots were counted under inverted microscope (Olymps, Tokyo, Japan). The number of ASC was the mean number of immunospots deducting the mean number of each contrast.

Assay of saliva sIgA and gut sIgA by ELISA

Saliva was collected from immunized mice[37]. Gut washing fluids was collected after the last immunization one week (week 8). Each well was coated with 100 μl of *H. pylori* lasate solution at concentration of 50 μg/ml in 0.05 mol/L sodium carbonate-bicarbonate buffer (pH 9.6) overnight at 4 ℃. After suction and three washes in PBS-Tween, 100 μl of 2 % BSA solution in PBS was added to each well and further incubated for 1hour at 37 ℃. The initial dilution of sample (saliva and gut washing fluids) was 1:20. 100 μl diluted samples was added to wells and incubated for 1hour at 37 ℃. After three washes in PBS-Tween, 100 μl of biotinylated goat anti-mouse IgA incubated for 1hour at 37 ℃. After washing three times with PBS-Tween, 100 μl of the avidinperoxidase solution was added and incubated for 1 hour at 37 ℃. Then washed and added 100 μl substrate solution. The plates were incubated for 20 minutes at 37 ℃. The reaction was stopped by addition of 100 μl of 0.5 mol/L hydrochloric acid, and the color development was measured by plate reader set at 405 nm.

Statistical analysis

Data were analyzed using Student' s t test. Results were expressed as mean±standard error. Differences were considered significant for values $P<0.05$.

RESULTS

Properties and morphology of PELA-Hp microspheres

The properties of PELA-*Hp* microspheres prepared with

different inherent viscosity of PELA (ranged from 0.1271-0.3329 dL/g) are shown in Table 1. The properties of microspheres were examined under light microscope in particle size and by UV spectrophotometer in protein loading and encapsulation efficiency. The morphology of microspheres was examined under scanning electron microscopy. The morphology of MS-2 is shown Figure 1.

Table 1 The properties of the PELA-*Hp* microspheres

Lot	PELA[η] (dL / g)*	Mean dimeter(μm)	Protein loading (%,w / w)	Encapsulation efficiency (%,w / w)
MS-1	0.1271	3.87	5.06	77.6
MS-2	0.2083	3.20	5.53	82.2
MS-3	0.3054	3.53	5.40	74.9
MS-4	0.3329	4.05	5.27	79.5

*The inherent viscosity of PELA is measured in tetrahydrofuran at 25 ℃.

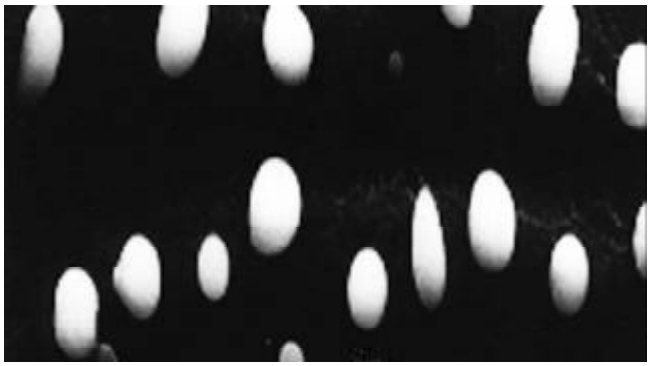

Figure 1 Scanning electron micrographs of MS-2 loaded *Hp*.

PELA-cystografin microsphere targeting

The images of the miniature pig by oral administration with PELA-cy*stografin* microsphere were observed by CT. The experiment could indirectly obtain the distribution of PELA microsphere in all gastrointestinal tract as time (day). The results suggested that PELA-*cystografin* microsphere mainly was distributed in the gastric cavity and at the surface of gastric mucosa after oral administration for three days. Then part of the microspheres gradually moved to enter the intestine by the stomach peristalsis and adhered to their surface (images not shown). After 15 days, the microspheres were found mainly distributed at the surface of intestine. The images are shown Figure 2.

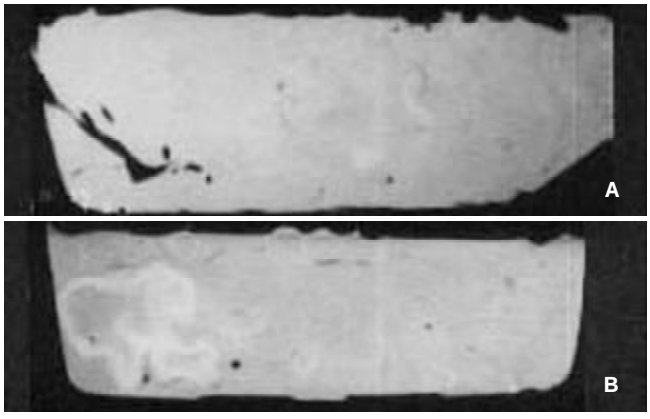

Figure 2 Images of pig stomach and gut after oral administration. (a) blank PELA microsphere; (b) PELA-*cystografin* microsphere.

Mucosal immune responses of orally immunized PELA-Hp microspheres

Saliva sIgA responses after oral immunization with soluble antigen, PELA-*Hp* microspheres with different inherent viscosities of PELA (ranged from 0.1271 to 0.3329 dL/g) were measured at week 3, week 5 and week 8 (Table 2). Immunization with soluble antigen induced weak saliva IgA antibodies. Immunization with all types of PELA-*Hp* microspheres elevated saliva sIgA level at week 3 (approximately 3-4 times higher than those with soluble antigen) and greatly enhanced after boosting (week 8). The changes of saliva sIgA at different stages after oral immunization related to the properties of PELA.

Table 2 Comparison of sIgA titres in saliva at different stages after oral immunization ($\bar{x}\pm s$)

Groups	Week 3	Week 5	Week 8
PBS	0.081±0.026	0.074±0.023	0.080±0.011
Ag	0.197±0.177	0.199±0.093	0.225±0.029
MS-0	0.090±0.041	0.071±0.013	0.110±0.046
MS-1	0.416±0.183	0.567±0.136	0.891±0.215
MS-2	0.399±0.179	0.608±0.209	0.882±0.264
MS-3	0.346±0.126	0.674±0.128	0.957±0.238
MS-4	0.304±0.105	0.523±0.211	0.944±0.143

At one week after last immunization with all types of PELA-*Hp* microspheres (week 8), the specific sIgA-ASCs,IgG-ASCs and sIgA were estimated by ELISPOT and ELISA.The results are shown in Table 3.

Table 3 Immune response induced with PELA-*Hp* microspheres at week 8 (n=20, $\bar{x}\pm s$)

Groups	PP sIgA-ASCs	(1×10^4) IgG-ASCs	Stomach sIgA-ASCs	(1×10^3) IgG-ASCs	Gut sIgA
PBS	0.13±0.04	0.16±0.07	0.19±0.08	0.23±0.06	0.234±0.08
Ag	0.27±0.11	0.29±0.09	0.35±0.11	0.36±0.15	0.34±0.17
MS-0	0.16±0.07	0.17±0.05	0.15±0.06	0.21±0.07	0.25±0.08
MS-1	6.58±1.38	3.78±0.82	4.17±1.09	0.74±0.29	1.75±0.46
MS-2	6.92±1.77	4.02±0.93	3.80±1.34	1.02±0.44	1.85±0.38
MS-3	6.77±1.46	3.56±1.10	4.12±0.83	1.81±0.73	1.62±0.81
MS-4	5.92±1.92	3.47±0.82	3.65±1.04	1.88±0.23	1.71±0.70

DISCUSSION

The relationship between the properties of PELA-*Hp* microspheres and molecular weight of PELA copolymer are shown Table 1. All types of PELA-*Hp* microspheres prepared with the same component PELA, whose protein loading, encapsulation efficiency and size were 5.06-5.40 %, 74.9-82.2 % and 3.20-4.05 μm, respectively, were not significantly different This confirmed the previous investigations[38]. Morphology of PELA microspheres using double emulsion evaporation method were investigated under sanning electron microscopy. The obtained microspheres were all spherical, homogeneous particles and had smooth surface (as shown in Figure 1). The results coincided with the report of SY Kim *et al*[39], while Kofler *et al*[40] reported that the microspheres were

porous particles with a rough surface. This is likely that different processing and manufacturing conditions of microspheres (such as o/$w_{out\text{-}water\ phase}$, $w_{inter\text{-}water\ phase}$ /o, stirring velocity, temperature etc), which result in morphologies of microspheres.

The images of the miniature pigs by oral administration of PELA-*cystografin* microspheres are shown in Figure 2. This indirectly clarified the conclusion arrived by Eldridge *et al*[41], which was that microspheres (mean diameter <10 μm) after oral administration firstly adhered to the surface of the gastrointestinal mucous membrane, then arrived at their Peyer' s patches (PP) to induce mucosal and systemic immune responses. Therefore, our obtained microspheres (mean diameter <5 μm) could induce mucosal and systemic immune responses by particle uptake into the PP. After oral administration, all types of PELA-*Hp* microspheres (week 8), the specific sIgA-ASCs, IgG-ASCs and sIgA in gut washing fluids obviously rose by ELISA and ELISPOT. The specific sIgA-ASCs and IgG-ASCs were approximately 5-9 times that with soluble antigen. ASCs in the intestine were more than those in stomach, and the majority of the ASCs were sIgA-ASCs. This result indicated that there appeared to be a good correlation between sIgA level in gut washing fluid and sIgA-ASCs in intestinal PP. The sIgA in gut washing fluid was about 3-6 times higher than those with soluble antigen (as shown in Table 3), that is very important for oral immunization to effectively induce mucosal immune response and protect against *Hp* infection[21,35]. The protein release of PELA-*Hp* microspheres was influenced by several parameters including PELA copolymer molecular weight, diameter, the ratio of lactide/PEG and the manufacturing technology. Immunization with all types of PELA-*Hp* microspheres elevated the saliva sIgA level at week 3 (approximately 3-4 times higher than those with soluble antigen) and greatly enhanced after boosting (week 8) (Table 2). At the same components of PELA (feed ratio of lactide/PEG=95:5 in this study), after oral immunization for 3 weeks, mice with PELA-*Hp* microspheres prepared with lower molecular weight of PELA , the saliva sIgA level became higher than the mice with PELA-*Hp* microspheres prepared with higher molecular weight of PELA. However, after oral immunization for 8 weeks, there was no differences in antibody production level between those formulations. This result can be explained as follows: the initial burst release of *Hp* was bigger from PELA-*Hp* microspheres prepared with lower molecular weight PELA, meanwhile because low molecular weight of PELA was degraded easily and can not effectively protect *Hp* release from microspheres ($t_{1/2}$ was short)[36], this means that mice orally immunized with PELA-*Hp* microspheres prepared with lower molecular weight PELA induced stronger mucosal and systemic immune responses after oral immunization in earlier stages. All types of PELA-*Hp* microspheres (η=0.1271-0.3329 dL/g PELA, 1-2 month degradation *in vitro*) were degraded fully and released encapsulated antigens at week 8 irrespective of differences of copolymer molecular weights. We also found in this study that antigen release from microspheres can be controlled and accommodated by dictating the component and molecular weight of polymer. This will play an important role in designing oral vaccines for the protection against diseases in the future.

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