

Uptake of cyclosporine A loaded colloidal drug carriers by mouse peritoneal macrophages *in vitro*¹

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KEY WORDS cyclosporins; microspheres; capsules; emulsions; peritoneal macrophages

ABSTRACT

AIM: To investigate the uptake of cyclosporine A loaded colloidal drug carriers by mouse peritoneal macrophage (MPM) *in vitro*. **METHODS:** The [³H]cyclosporine A loaded colloidal particles: polylactic acid nanospheres, polylactic acid nanocapsules, and microemulsions were prepared. The [³H]cyclosporine A loaded colloidal particles were incubated with MPM for 30 min at 37 °C, then the cells were separated from the colloidal particles and the radioactivity was measured by a liquid scintillation counter. **RESULTS:** In comparison to the cyclosporine A solution, the binding to polylactic acid nanospheres produced a 20-fold increase in the uptake of cyclosporine A by MPM in 30 min incubation, whereas some obvious decrease in the uptake of cyclosporine A by MPM was observed in the binding of cyclosporine A with polylactic acid nanocapsules or microemulsions. The surfactant coating and plasma protein adsorption were found to have marked effects on the uptake of cyclosporine A loaded nanospheres by MPM. **CONCLUSION:** Our present study indicated that colloidal drug carriers might affect the targeting of cyclosporine A to mononuclear phagocyte system.

INTRODUCTION

Cyclosporine A (CyA), a lipophilic endecapeptide consisting of 11 amino acids, has been used in the treatment of solid organ and marrow transplantation to avert graft rejection since 1978. Its mode of action is still un-

clear, but its main target appears to be the T lymphocyte⁽¹⁾. CyA can mediate immunosuppression of primary cytotoxic T cell responses by selectively impairing the release of interleukin-1 from macrophages and that of interleukin-2 from activated T helper cells⁽²⁾. The successful delivery of macromolecular drugs is often problematic. Protein and peptide drugs generally can not be effectively absorbed by oral administration, since they are liable to be hydrolyzed or denatured in the gastrointestinal tract. Parenteral administration also poses some problems, for these drugs are usually quickly metabolized and eliminated from the blood stream. One of the current approaches to achieve a high bioavailability of proteins or peptides utilizes the use of novel drug delivery carriers, such as liposomes, nanospheres, nanocapsules, and microemulsions⁽³⁻⁵⁾. It was found that most of these colloidal particles were quickly to be recognized by the mononuclear phagocytic system and the polymorphonuclear leukocytes and are targeted to these phagocyte cells which are also the main target sites of CyA⁽⁶⁻⁸⁾. However, few studies related to the investigation on the uptake of CyA loaded colloidal carriers by phagocyte cells have been reported, which is assumed to be closely relevant to the immunosuppressive effect of CyA. In the present study, we prepared three kinds of [³H]CyA loaded colloidal particles: polylactic acid (PLA) nanospheres, polylactic acid nanocapsules, and microemulsions. The mouse peritoneal macrophages (MPM) were used as a cell model to investigate the uptake of colloidal particles loading CyA by cells *in vitro*. The possible mechanism and factors affecting the uptake were also discussed.

MATERIALS AND METHODS

Drugs and reagents CyA was obtained from Sichuan Industrial Institute of Antibiotics (Chengdu, China); [³H]CyA with a specific radioactivity of 3.26 × 10¹¹ Bq/g and a radiochemical purity over 94 % was provided by China Institute of Atomic Energy (Beijing, China); Polylactic acid (PLA) with the molecular weight of

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15 000 was obtained from Shandong Medicinal Products (Jinan, China); *L*- α -lecithin, Pluronic F₆₈, Cremophor RH 40, Brij 78, Myrj 53, and Myrj 59 were purchased from Sigma (St Louis, MO); Triton X-100 was purchased from FARCO (Chemical Supplies, Hong Kong); 2,5-Diphenyloxazole (PPO) and 1,4-bis(5-phenyl-2-oxazolyl)-benzene (POPOP) were provided by Fluka (Buchs, Switzerland); All other chemicals were of analytical reagent grade.

Instruments Liquid Scintillation Counter, Pharmacia WALAC 1410, Turku, Finland; Submicro Particle Sizer, Model 370, Santa Barbara, California, USA.

Mice KM mice (20 g \pm 2 g, Grade II, Certificate No 01-3049) of both sexes were obtained from the Department of Experimental Animals, Beijing University Health Science Center.

Preparation of CyA loaded colloidal particles

The CyA loaded PLA nanospheres and nanocapsules were prepared by the method of emulsion-evaporation^[3]. For the CyA loaded PLA nanospheres, 50 mL acetone solution containing 50 mg CyA (with [³H]CyA) and 450 mg PLA were injected into 150 mL water containing 375 mg Pluronic F₆₈ at 0–2 °C under 60 \times g agitation. Thereafter, the mixture was evaporated at 60 °C in a bath 50 mL. For the CyA loaded PLA nanocapsules, 50 mL acetone solution (in bath at 70 °C) containing 50 mg CyA (with [³H]CyA), 100 mg *L*- α -lecithin, 0.5 mL oil, and 350 mg PLA were injected into 150 mL water (bathed at 70 °C) containing 375 mg Pluronic F₆₈ under 60 \times g agitation. Then the mixture was evaporated at 60 °C in a bath 50 mL. The CyA loaded microemulsions were prepared with the Cremophor RH 40 as a surfactant, 1,2-propylene glycol and ethanol as co-surfactants. The particle size of each kind of colloidal particles was measured by a Submicro Particle Sizer. The details and abbreviated descriptions of the tested samples are list-

ed in Tab 1.

Preparation of MPM suspension MPM was prepared as described previously^[9]. Briefly, six KM mice were killed by cervical dislocation, then each mouse was peritoneally injected with 4 mL cool D-Hanks' solution. The peritoneum of the mouse was massaged for 1 min and the solution inside the abdominal cavity was removed, pooled, and mixed homogeneously. A sample was taken for the cell counting and the final cell suspension was diluted to a concentration of 1 \times 10⁸ cells/L by adding RPMI-1640 medium containing 5 % fetal calf serum.

Uptake of [³H]CyA loaded colloidal particles by MPM *in vitro* Tested sample 0.5 mL and 1.0 mL of MPM suspension were added together in a 5-mL centrifugation tube. Then the tubes were maintained at 37 °C for 30 min and shaken at 5-min intervals. After incubation the suspension was placed on ice to stop the uptake and then centrifuged at 60 \times g for 5 min to separate cells from nanospheres, nanocapsules, or microemulsions. The supernatant was discarded and the cell pellet was resuspended and washed twice with saline by centrifugation. A parallel protocol with the MPM suspension only was carried out as the cell control.

Scintillation counting The cell precipitate obtained in each tube was digested for 1.5 h at 75 °C in a solution containing 80 μ L formic acid and 20 μ L oxydol. Bray scintillation fluid (5 mL) was added and mixed. After standing for 1 h, the radioactivity (dpm) was measured by a scintillation counter.

Another operation with 0.5 mL test samples only was performed at the beginning of the digestion and the result was taken as the [³H]control. In order to avoid the possible variance that may result from the poor separation of the cells and the colloidal particles, a parallel protocol with each test sample only was carried out at the

Tab 1. Codes and the related data of test samples.

Sample	Stabilizer	Particle size/ nm	Radioactivity concentration/ 1 \times 10 ⁻⁸ Bq \cdot L ⁻¹	CyA concentration/ g \cdot L ⁻¹
[³ H]CyA-NSC	Pluronic F ₆₈	59 \pm 11	2.6	1.0
[³ H]CyA-NCC	Pluronic F ₆₈	82 \pm 37	2.6	1.0
[³ H]CyA-MEC	RH 40	22 \pm 6	2.6	1.0
[³ H]CyA-Sol	Tween 80		2.6	1.0

CyA, Cyclosporine A; NSC, Nanospheres colloid; NCC, Nanocapsules colloid; MEC, Microemulsions colloid; Sol, Solution; RH 40, Cremophor RH 40.

very beginning of the scintillation counting in each study and the radioactivity (dpm) from each parallel protocol was deduced from the uptake value (dpm) of each test sample.

Blood protein adsorption on the surface of colloidal particles Mouse plasma was added into test sample after it was prepared. Then test sample was homogenized by shaking at 37 °C for 5 min. After blood protein adsorption, the *in vitro* uptake test was conducted as described above.

Surfactants coating on the surface of colloidal particles Three kinds of surfactants; Brij 78, Myrj 53, and Myrj 59 were used to modify the surface of the colloidal drug carriers. The surfactant was added into the test samples and then homogenized under ultrasonic condition for 5 min⁽⁹⁾. After surfactant coating, the *in vitro* uptake test was conducted as described above.

Statistical analysis Results were expressed as $\bar{x} \pm s$ and analyzed by *t*-test to compare the different uptake between test samples.

RESULTS

The uptake of CyA loaded colloidal particles by MPM of different test samples As shown in Tab 2, the incubation of the cells with nanospheres had a great increase in uptake and the cells with nanocapsules or microemulsions had decreased uptake, respectively. In comparison with [³H]CyA-Sol, a 20-fold increase in uptake by the MPM was observed when CyA was bound to nanospheres. However when CyA was bound to nanocapsules or microemulsions, about 27.2 % and 51.0 % decrease in the uptake by MPM was observed, respectively. The average radioactivity value (dpm) of [³H] control was 46581240 ± 3611453. The value of control was less than 1000 in each study.

Tab 2. *In vitro* uptake of [³H]CyA loaded colloidal particles by mouse peritoneal macrophage at 37 °C. n = 5. $\bar{x} \pm s$. *P < 0.05, °P < 0.01 vs [³H]CyA-Sol.

Sample	dpm (after 30 min)	Change/%
[³ H]CyA-NSC	457026 ± 23660°	+ 2000
[³ H]CyA-NCC	15820 ± 1380 ^b	- 27.2
[³ H]CyA-MEC	10640 ± 1287 ^b	- 51.0
[³ H]CyA-Sol	21733 ± 5087	0.0
[³ H]Control	46581240 ± 3611453	
Cell control	940 ± 247	

Effect of blood protein adsorption on the uptake As shown in Tab 3, the blood protein adsorption studies demonstrated an increase in the uptake of CyA by MPM when the amount of the mouse plasma had increased to 40 μL in the 0.5 mL test sample. Compared with the [³H]CyA-NSC in the absence of plasma, there was no markedly different uptake of CyA by MPM adding 20 μL plasma to the 0.5 mL tested sample. A 1.29-fold increase in uptake by MPM was found when the plasma added to the test samples increased to 40 μL.

Effect of surfactant coating on the uptake As shown in Tab 4, compared with [³H]CyA-NSC without surfactant coating, all kinds of surfactants used to modify the surface of the nanospheres greatly decreased the uptake of CyA by MPM. In comparison with the nanospheres without surfactant coating, 76.5 %, 91.7 %, or 96.5 % decrease in uptake by MPM was observed when the surface of nanospheres was coated by Brij 78, Myrj 53, or Myrj 59, respectively. The results also demonstrated a good relationship between the HLB value of surfactant and the ability of the surfactant to decrease the uptake. When the HLB value of the coating surfactant increased, the uptake of CyA loaded nanospheres decreased.

Tab 3. Effect of blood protein adsorption on *in vitro* uptake of [³H]CyA loaded nanospheres by mouse peritoneal macrophage at 37 °C. n = 5, $\bar{x} \pm s$. *P > 0.05, °P < 0.01 vs [³H]CyA-NSC with no plasma. φ means percent of volume.

Sample	Plasma of mouse (μL)	Dpm (after 30 min)	Change/%
[³ H]CyA-NSC	0.0	457020 ± 23660	0.0
[³ H]CyA-NSC	20.0 (φ = 4 %)	434473 ± 110366°	- 4.9
[³ H]CyA-NSC	40.0 (φ = 8 %)	1045260 ± 111493°	+ 129
Cell control		880 ± 187	

Tab 4. Effect of surfactant coating on *in vitro* uptake of [³H]CyA loaded nanospheres by mouse peritoneal macrophage at 37 °C. *n* = 5. *x* ± *s*. **P* < 0.05, †*P* < 0.01 vs [³H]CyA-Sol. ‡*P* < 0.01 vs [³H]CyA-NSC without surfactant coating. HLB: hydrophile-lipophile balance.

Sample	Surfactant (HLB)	Surfactant concentration/%	dpm (after 30 min)	Change vs control/%	Change vs [³ H]CyA-Sol/%
[³ H]CyA-NSC	None (control)		457026 ± 23660 [†]	0.0	+ 2000
[³ H]CyA-NSC	Brij 78 (15.3)	2.0	107486 ± 12080 ^{††}	- 76.5	+ 390
[³ H]CyA-NSC	Myrj 53 (17.9)	2.0	38000 ± 9253 ^{†††}	- 91.7	+ 74.8
[³ H]CyA-NSC	Myrj 59 (19.1)	2.0	16113 ± 3413 ^{††}	- 96.5	- 25.8
[³ H]CyA-Sol			21733 ± 5086		0.0
Cell control			787 ± 173		

DISCUSSION

On the basis of the results presented in the experiment, we found that on binding CyA with nanospheres the uptake was increased, while on binding CyA with nanocapsules or microemulsions the uptake was decreased. The increased uptake of CyA loaded nanospheres was possibly due to the rapid uptake of the nanospheres by macrophages^[10,11]. The decreased uptake of CyA loaded in nanocapsules or microemulsions may be probably due to the soft surface of the particles and the large amounts of surfactants used in preparation of these two colloidal carriers^[9]. From the results of the surfactant coating experiment, we can conclude that the surfactant coating can surely decrease the uptake by macrophages. It is generally assumed that the rapid colloidal carrier phagocytosis was mediated by the adsorption of blood components onto the surface of the particles^[6-8]. The interactions between the colloidal particles and the blood components are complex. These components might be reversibly or irreversibly adsorbed on the surface of the particles, and might be replaced by others. The analysis of plasma protein adsorbed on the colloidal particles has been established using two-dimensional polyacrylamide gel electrophoresis^[12]. The relevance of the adsorbed plasma to the *in vivo* fate of the colloidal particles still remains unclear. The results of present study showed that the plasma adsorption can strongly activate phagocytosis by macrophages (Tab 3).

In the case of colloidal carriers, the rapid uptake by MPS may be partially retarded by coating the particles with surfactants. In fact, the use of hydrophilic coatings to decrease uptake of colloidal particles by the MPS has become an important recent development. It was pro-

posed that when the hydrophilicity of surfactants coated on the surface of the colloidal carriers increased, the uptake of the carriers by MPS decreased^[13]. The results presented here showed that when the hydrophile-lipophile balance (HLB) value of the surfactants increased, the uptake of CyA loaded in nanospheres was decreased (Tab 4). Brij78, Myrj 53, and Myrj 59 are surfactants containing blocks of poly ethylene glycol(PEG). It is proposed that the flexibility and rapidly changing structure of the PEG chains made the MPS difficult to recognize the colloidal particles^[14]. The molecular weights of PEG blocking these surfactants are: Brij 78 with PEG 1000, Myrj 53 with PEG 2000, and Myrj 59 with PEG 5000. A direct relationship has been observed between the length of PEG chain on the particle surface and the uptake by macrophages. The uptake by MPM decreased with increasing PEG molecular weight (Tab 4).

In conclusion, colloidal drug carriers can affect the targeting of CyA to mononuclear phagocytic system. This targeting can be affected by plasma adsorption and can be controlled by changing the surface character of the particles.

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小鼠腹腔巨噬细胞对环孢菌素 A 胶体型药物载体的体外摄取¹

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关键词 环孢菌素类; 微球体; 胶囊; 乳状剂; 腹腔巨噬细胞

目的: 研究小鼠腹腔巨噬细胞体外对环孢菌素 A 胶体亚微粒的摄取. **方法:** 制备 [³H]-环孢菌素 A 纳米球、纳米囊和微乳三种胶体亚微粒, 以小鼠腹腔巨噬细胞为体外细胞模型, 研究小鼠腹腔巨噬细胞体外对环孢菌素 A 胶体亚微粒的摄取. **结果:** 纳米球可使巨噬细胞对环孢菌素 A 的摄取达对照溶液的 20 倍, 而纳米囊和微乳则使巨噬细胞对环孢菌素 A 的摄取明显减少. 在胶体亚微粒的表面进行表面活性剂修饰和血浆蛋白吸附对巨噬细胞的摄取有明显影响. **结论:** 将环孢菌素 A 包封于胶体亚微粒中能改变其对巨噬细胞的靶向作用.

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