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Increase in gentamicin uptake by cultured mouse peritoneal macrophages and rat hepatocytes by its binding to polybutylcyanoacrylate nanoparticles

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Abstract

Polybutylcyanoacrylate nanoparticles of ^{3}H labelled gentamicin were used to investigate the possibility of gentamicin nanoparticles as the drug delivery system for intracellular chemotherapy. ^{3}H labelled gentamicin nanoparticles were incubated with mouse peritoneal macrophages or rat hepatocytes for various lengths of time. The cells were then separated from the nanoparticles and the radioactivity of ^{3}H in the cells was measured by a liquid scintillation counter. By comparison with the gentamicin solution, the binding to nanoparticles produced a 5.34-fold increase in the uptake of gentamicin by the macrophages in 30-min incubation and 26.74-, 8.03- and 7.36-fold increase in uptake by the hepatocytes in 1-, 12- and 24-h incubation. The stabilizers used in the preparation of nanoparticles, particle size, surfactant coating and the gentamicin concentration were each found to have an effect on the uptake of the nanoparticles by both types of cells.

Keywords: Gentamicin; Nanoparticles; Mouse peritoneal macrophages; Rat hepatocytes; In vitro cell uptake

1. Introduction

Infections caused by intracellular microorganisms are difficult to treat because bacteria inside the phagosomes are protected from antibiotics (Johnson et al., 1980; Trouet and Tulkens, 1981; Brajtburg et al., 1990). The need for intracellular targeting of antibiotics has been recognized for many years(Tulkens, 1985) and has become more urgent because intracellular infections are now often associated with AIDs. Colloidal drug carri-

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Table 1
Gentamicin samples tested: nanoparticle-bound and in solution

Sample[a]	Stabilizer used	Particles size (nm)	Radioactive concentration ($\times 10^5$ Bq/ml)	Gentamicin concentration (mg/ml)	Sample abbreviation
^{3}H-GM-NP, colloid	Dextran 70	1550 ± 247	2.62	2.0	LNP-D
^{3}H-GM-NP, colloid	Pluronic F68	448 ± 170	2.62	2.0	LNP-F
^{3}H-GM-NP, colloid	Dextran 70	69 ± 17	2.62	2.0	SNP-D
^{3}H-GM-NP, colloid	Pluronic F68	245 ± 39	2.62	2.0	SNP-F
^{3}H-GM-NP, FD	Dextran 70	68 ± 13	2.62	2.0	FD-SNP-D
^{3}H-GM-Solution			2.62	2.0	GM-Sol

^{3}H-GM-NP, hydrogen-3-labelled gentamicin nanoparticles; GM, gentamicin; NP, nanoparticles; FD, freeze-dried and reconstituted before use; LNP, large NP; SNP, small NP; Sol, solution; D, Dextran 70; F, Pluronic F68.
[a] The ^{3}H-GM-NP samples were prepared by emulsion polymerization with α-butylcyanoacrylate.

ers, such as liposomes and nanoparticles, are efficiently taken up by phagocytic cells of the body(Kreuter, 1983; Lenaerts et al., 1984) and exhibit strong tendency to accumulate in the lysosomes (Couvreur et al., 1977). Thus, the colloidal drug carriers with antibiotics hold promise for the treatment of intracellular infections.

In general, nanoparticles (NP) offer advantages over liposomes due to their relatively easy production methods and long shelf-lives (Fattal et al., 1991). In the research of NP as drug delivery system, it has been shown, for example, that ampicillin-loaded polyisohexylcyanoacrylate nanoparticles gave effect against *L. monocytogenes* and *Salmonella typhimurium* (Youssef et al., 1988; Fattal et al., 1989). The resistance of intracellular infection to chemotherapy seems to be related to the low intracellular delivery of commonly used antibiotics.

In the present study, therefore, the ^{3}H-labelled gentamicin polybutylcyanoacrylate nanoparticles (^{3}H-GM-NP) were prepared, and the effectiveness of these nanoparticles in increasing the intracellular delivery of gentamicin and some relevant factors that may affect this procedure were studied using mouse peritoneal macrophages and rat hepatocytes as the model cells since nanoparticles (80–200 nm diameter) are mostly localized in the macrophage (Kupffer cells) of the liver after i.v. administration to mice or rats.

2. Materials and methods

2.1. Materials

^{3}H-labelled gentamicin with a specific radioactivity of 3.26×10^8 Bq/mg and a radiochemical purity over 93% was labelled by Department of Isotopes, Chinese Institute of Atomics (Beijing, China). Butylcyanoacrylate was obtained from Shenzen South Medical Gum Co. (Shenzen, China), Dextran 70 from Pharmacia (Uppsala, Switzerland), Pluronic F68 from Sigma (St. Louis, MO), 2.5-diphenyloxazole (PPO) and 1.4-bis(5-phenyl-2-oxazolyl)-benzene (POPOP) from Fluka (Buchs, Switzerland) and Gentamicin (626 μ/mg) from Sichuan Pharmaceutical Co. (Chengdu, China). All other chemicals were commercially available products of special reagent grade.

2.2. Preparation of tested sample

^{3}H-GM-NP with different particle sizes and stabilizers were prepared by emulsion polymerization (Zhang and Liao, 1996), with the α-butylcyanoacrylate as the carrier material. To investigate the behaviour of the drug-loaded nanoparticles, gentamicin was added, to a concentration of 2.0 mg/ml. The details and abbreviated descriptions of the tested samples are listed in Table 1.

2.3. Preparation of mouse peritoneal macrophage suspension

Five 20 ± 2 g KM mice (Animal Centre of West China University of Medical Sciences, Chengdu, China) were killed by cervical dislocation, then each was peritoneally injected with 2 ml cool Hank's solution. The peritoneum of the mouse was massaged for 1 min and the solution inside of abdominal cavity was drawn out, pooled and mixed homogeneously. A sample was taken for the cell counting and the final suspension was dissoluted to 1×10^5/ml with RPMI-1640 medium containing 5% fetal calf serum.

2.4. Preparation of rat hepatocyte suspension

The livers of Wistar rats (200 ± 20 g, 5 weeks, male) (Animal Centre of West China University of Medical Sciences, Chengdu, China) was excised and perfused with Hank's solution first, then perfused with 0.1% collagenase solution adjusted to pH 7.4 by Hepes and kept in a 37 ± 1°C water bath for 30-min. Cells were filtered on 200-mesh gauze and collected by centrifugation. The Cell suspension was washed three times by adding 10-ml Tris–NH_4Cl solution each time, kept in a 37 ± 1°C water bath for 10-min and centrifuged. Next, RPMI-1640 medium containing 5% fetal calf serum, 10 μg/ml bovine insulin and 0.4 μg/ml dexamethasone was added, to a density of 3.5×10^5 cells per ml. Rat hepatocytes were used after 24 h of culture under 37°C and 5% CO_2 95% air-humidified atmosphere.

2.5. In vitro uptake by mouse peritoneal macrophages

Mouse peritoneal macrophage (1.0 ml) suspension (1×10^5 cells) and 0.5-ml tested sample (1.31×10^5 Bq) were added to a 5-ml centrifugation tube, and the tubes were then maintained at 37 ± 1°C for 30-min and shaken at 5-min intervals. After the suspending of the phagocytosis by immersion in an ice bath, the cells were separated from nanoparticles by centrifuging (1000 rpm, 5-min), discarding the supernatant, washing with 3-ml saline and repeating this procedure for two more times. A parallel protocol with the mouse peritoneal macrophages only was done as the cell control.

2.6. In vitro uptake by rat hepatocytes

The 24-well culture plate (l5 mm) was used. Rat hepatocyte (1.0 ml) suspension (3.5×10^5 cells), 0.5-ml tested sample (1.31×10^5 Bq) and 0.5-ml RPMI-1640 medium containing 5% fetal calf serum were added to each well and mixed. The culture plates were maintained in a 37°C and 5% CO_2 95% air-humidified atmosphere for 1-, 12- or 24-h, following which the culture in each well was transferred to a 5-ml centrifugation tube, and each well was washed with 3-ml saline which was also transferred to the same tube. The cells were separated from the nanoparticles by the above mentioned technique. A parallel protocol with the rat hepatocytes only was done as the cell control.

2.7. Scintillation counting

The cell sediment obtained in each tube was digested for 1-h at 80°C in a solution containing 80-μl formic acid and 20-μl oxydol. Bray scintillation fluid was added and mixed (5 ml). After standing for 1 h, the radioactivity (CPM) was analyzed in a scintillation counter.

Another operation with 0.5-ml GM-Sol only was performed, beginning at digestion and the result was taken as the ^{3}H control. In order to check the efficient separation of the cells and nanoparticles, and avoid the possible variance that may result from the poor separation, the parallel protocol with each ^{3}H-GM-NP sample only was carried out from the very beginning to the scintillation counting in each study and the radioactivity (CPM) from each parallel protocol was deducted from the uptake value for each ^{3}H-GM-NP sample.

3. Results

As shown in Figs. 1 and 2, the uptake of gentamicin by mouse peritoneal macrophages and rat hepatocytes was significantly increased by

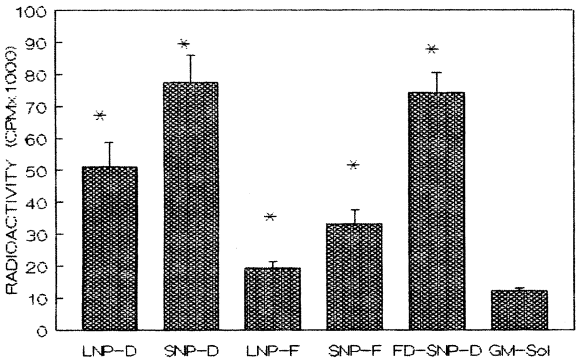

Fig. 1. Radioactivity of ^3H in mouse peritoneal macrophages after 30-min incubation at 37°C with ^3H-labelled gentamicin nanoparticles (for sample abbreviations, see Table l). Date are the mean ± S.D. of $n = 5$ each sample. * $P < 0.001$ vs. GM-Sol.

binding gentamicin to nanoparticles, in comparison with the gentamicin solution. The sequence in which the nanoparticle samples increased the endocytosis of gentamicin by mouse peritoneal macrophages are as follows: SNP-D, FD-SNP-D > LNP-D > SNP-F > LNP-F, while that by rat hepatocytes was SNP-D, FD-SNP-D > LNP-F, SNP-F. The differences in the uptake level between SNP-D and FD-SNP-D by two types of cells, as well as the difference in the uptake value by rat hepatocytes between LNP-F and SNP-F, were not significant ($P > 0.05$), while the differences among all the other ^3H-GM-NP samples were significant ($P < 0.001$). In comparison with GM-Sol, a 5.34-fold increase in uptake by the mouse peritoneal macrophages after 30-min incubation, and 26.73-, 8.03- and 7.36-fold increase in uptake by the rat hepatocytes after 1-, 12- and 24-h incubation were observed when the formulation of SNP-D was tested. The average radioactivity value (CPM) of ^3H control is 6 797 196 ± 564 167. The value of each ^3H-GM-NP is only less than 500 and the value of cell control is less than 200 in each study.

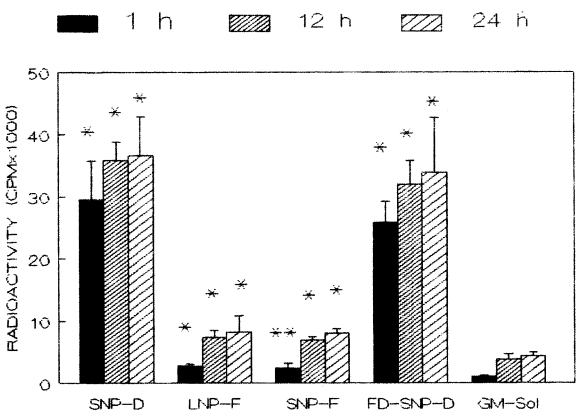

Fig. 2. Radioactivity of ^3H in the rat hepatocytes after 1-, 12- and 24-h incubation at 37°C under 5% CO_2 with ^3H-labelled gentamicin nanoparticles. Date are the mean ± S.D. of $n = 5$ each sample. * $P < 0.001$, ** $P < 0.01$ vs. GM-Sol.

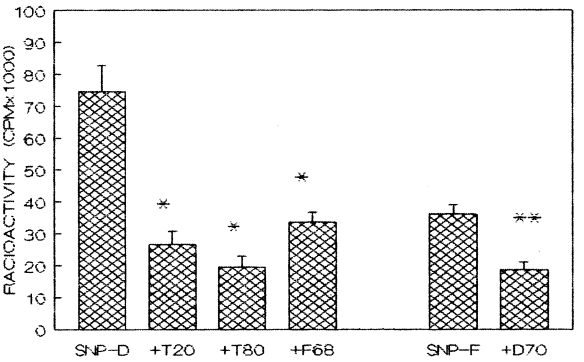

Fig. 3. Effect of surfactant coating on in vitro uptake of ^3H-gentamicin nanoparticles by mouse peritoneal macrophages after 30-min incubation at 37°C ($n = 5$). Surfactant was added after the preparation of each sample and each sample was then homogenized ultrasonically for 5-min. The surfactant concentrations were 1.5% (w/v). T20, Tween 20; T80, Tween 80; F68, Pluronic F68; D70, Dextran 70. Date are the mean ± S.D. of $n = 5$ each sample. * $P < 0.001$ vs. SNP-D, ** $P < 0.001$ vs. SNP-F.

Different surfactants were added to some ^3H-GM-NP samples, respectively, which were then homogenized ultrasonically for 5-min and the in vitro uptake test was then conducted as described above. The results were summarized in Figs. 3 and 4. The uptake values of SNP-D by two types of cells decreased after coating with each of the three surfactants tested, in which the Tween 80 produced the greatest decrease and Pluronic F68, the least. Since Dextran 70 had a favourable effect on cell uptake in the previous test, SNP-F was coated with Dextran 70 to determine whether this would produce an increase in endocytosis; however, the uptake level by mouse peritoneal macrophages decreased rather than increased

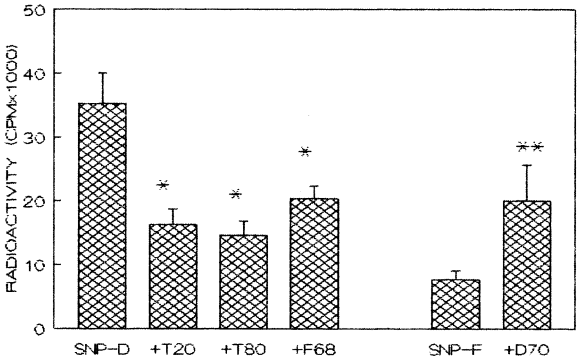

Fig. 4. Effect of surfactant coating on in vitro uptake of ^{3}H-gentamicin nanoparticles by rat hepatocytes after 24-h incubation at 37°C under 5% CO_2 ($n = 5$). Surfactant was added after the preparation of each sample and each sample was then homogenized ultrasonically for 5-min. The surfactant concentrations were 1.5% (w/v). T20, Tween 20; T80, Tween 80; F68, Pluronic F68; D70, Dextran 70. Date are the mean ± S.D. of $n = 5$ each sample. * $P < 0.001$ vs. SNP-D, ** $P < 0.001$ vs. SNP-F.

($P < 0.001$), while the uptake level by rat hepatocytes did increase by 159% ($P < 0.001$).

Different amounts of gentamicin were added to SNP-D samples and after a thorough shaking of each sample, the in vitro uptake experiment was carried out as described above. The results are given in Figs. 5 and 6. The uptake level of SNP-D

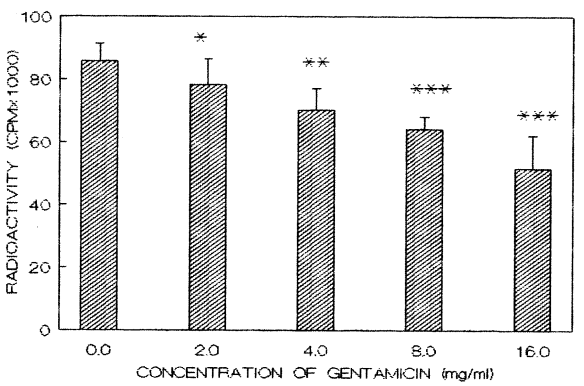

Fig. 5. Effect of gentamicin concentration on the in vitro uptake of ^{3}H-gentamicin nanoparticles (SNP-D) by mouse peritoneal macrophages at 37°C ($n = 5$).Gentamicin was added after the preparation of the samples and the samples were then shaken thoroughly. Date are the mean ± S.D. of $n = 5$ each sample. * $P > 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. 0.0 (no GM added).

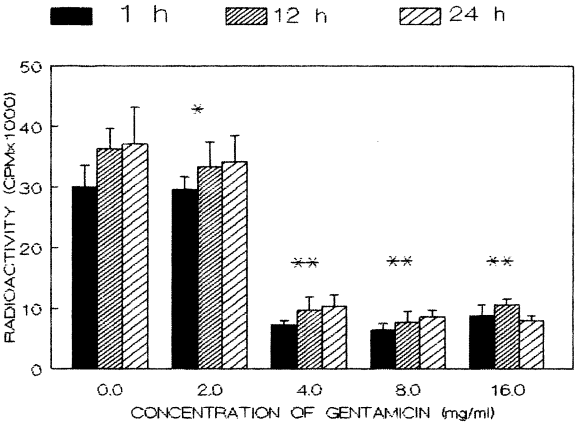

Fig. 6. Effect of gentamicin concentration on the in vitro uptake of ^{3}H-gentamicin nanoparticles (SNP-D) by rat hepatocytes after 1-, 12- and 24-h incubation at 37°C under 5% CO_2 ($n = 5$). Gentamicin was added after the preparation of the samples and the samples were then shaken thoroughly. Date are the mean ± S.D. of $n = 5$ each sample. * $P > 0.05$, ** $P < 0.001$ vs. 0.0 (no GM added).

by two types of cells dropped, but not significantly ($P > 0.05$) with gentamicin 2.0 mg/ml and decreased significantly ($P < 0.001$) when the concentration of gentamicin was to 4.0 mg/ml or greater.

4. Discussion

It has been proved that the nanoparticles are mainly taken up by the macrophages in the reticuloendothelial system (Lenaerts et al., 1984). The mouse macrophages were chosen as the model cell in this study since the mice were used in our previous investigations for gentamicin nanoparticles, including body distribution, toxicity and pharmacodynamics. The hepatocytes were also utilized late because most of the macrophages are located in the liver and the hepatocytes might have the chance to take up the nanoparticles. Sophisticate skills are needed in the culture of hepatocytes and rat hepatocytes are relatively more easy to deal with. Of course, these two types of cells are quite different in size and particularly in the capacity of endocytosis, however, the purpose of this study is not to compare their differences.

The mechanism by which the nanoparticles increased the endocytosis of gentamicin in the present study may be based on the nanoparticles being taken up by the cells as 'foreign materials', consequently the gentamicin carried by the nanoparticles could be delivered into the cells. An interesting finding is that the nanoparticle samples were not treated equally by the two types of cells; that is, cells had different affinities for the nanoparticles.

The endocytosis was clearly affected by the stabilizers used in the nanoparticle formulations. Higher uptake values of gentamicin by two types of cells were recorded when Dextran 70 was used as the stabilizer ($P < 0.001$). For instance, the uptake level of SNP-D is higher than that of SNP-F (Figs. 1 and 2) and the uptake value of LNP-D was higher than that of LNP-F (Fig. 1), indicating that Dextran 70, which is homogeneously adsorbed on the surface of the nanoparticles was more favourable to the interaction between the nanoparticles and these two types of model cells. The affinity of Dextran to liver which was demonstrated (Kaneo et al., 1988) might explain this result.

The endocytosis was also influenced by the particle size, particularly in the test of mouse peritoneal macrophages. When the same stabilizer was applied, the smaller nanoparticle samples produced higher uptake values ($P < 0.001$). As seen in Fig. 1, the uptake value of SNP-D was larger than that of LNP-D and the uptake value of SNP-F was larger than that of LNP-F. However, the uptake values were close between LNP-F and SNP-F in the rat hepatocytes ($P > 0.05$). The effect of particle size of nanoparticles may not have been significant because rat hepatocytes are much larger than nanoparticles. In the endocytosis of the rat hepatocytes, the uptake level in the first 1-h increased markedly and continued to increase until 12-h ($P < 0.001$), but changed little in the subsequent 12-h ($P > 0.05$), suggesting that the rat hepatocytes were saturated.

The endocytosis of nanoparticles by the tested cells was affected by coating with hydrophilic surfactants, such as polysorbates and Pluronic F68, indicating that the surface chemical characteristics of nanoparticles may play an important role in their interaction with the cells. Since surfactants are often used in the preparation of nanoparticles, the choice of surfactant must be considered carefully. The uptake in the reticuloendothelial system could be controlled to some extent simply by coating the nanoparticles with the proper surfactant, if a higher drug concentration outside of the reticuloendothelial system is needed. However, the different effect of Dextran 70 on the endocytosis of SNP-F between mouse peritoneal macrophages and rat hepatocytes seems not to be understood. As we know, the Dextran is quite different from surfactant which could quickly appear in the intersurface of two phases. Dextran with a long chain demonstrates the characteristics of the hydrogel and adhere slowly on the surface of the particles. The preparation procedure of nanoparticles last several hours so the Dextran may have enough time to adsorb. The coating procedure was only 5-min and the endocytosis by mouse peritoneal macrophages lasted only 30-min, so it was deduced that the adherence of Dextran was not completed and the endocytosis of relatively small mouse peritoneal macrophages could be interfered by the long chain Dextran. While the uptake period of rat hepatocytes was 24-h and rat hepatocytes also is larger, so an increase in endocytosis seems logical. Since coating with Dextran 70 seems favourable to the uptake of nanoparticles by rat hepatocyte; its use may increase the drug delivery in the parenchymal cells of the liver.

It was previously demonstrated that the polybutylcyanoacrylate nanoparticles are negatively charged and the surface potential of this type of nanoparticles decreased when the positively charged gentamicin was added (Zhang et al., 1996), indicating the adsorption of gentamicin molecules on the surface of polybutylcyanoacrylate nanoparticles. In this investigation, the gentamicin was added after the preparation of the nanoparticles, so molecules of gentamicin could associate with the nanoparticles only through the surface adsorption. With many NH_2 and OH groups, gentamicin belongs to the typical hydrophilic molecule. With the increase of gentamicin concentration, more and more gentamicin molecules may be adsorbed on the surface of the

nanoparticles, leading to changes in surface chemical properties. Since the hydrophilic nanoparticles do not interact well with the lipophilic cell membrane, the endocytosis of nanoparticles decreases, which suggests that the concentration of a hydrophilic drug should be controlled accordingly. The endocytosis may differ, if the drug molecules are not adsorbed on the surface of the nanoparticles, but are rather embedded in the whole matrix of nanoparticles, or loaded inside of the nanocapsules.

It was also noted that the pattern of Figs. 5 and 6 was different. The uptake of nanoparticles by mouse peritoneal macrophages decreased gradually with the increase of gentamicin concentration, while the uptake by rat hepatocytes dropped suddenly when gentamicin concentration changed from 2 to 4 mg/ml. One possible explanation might be the difference in endocytosis capacity for two types of cells. The effect of gentamicin concentration on the uptake of nanoparticles by rat hepatocytes was relatively larger because of its lower endocytosis capacity.

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