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Treatment of Experimental Salmonellosis in Mice with Streptomycin Entrapped in Liposomes

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Liposome-entrapped streptomycin (SM) was compared with free SM for therapeutic efficacy against experimental salmonellosis in mice. All of the mice infected with the virulent strain of *Salmonella enteritidis* 116-54 died between days 5 and 7, and a dose of 20 mg of free SM per kg administered 24 h after the bacterial inoculation did not prolong the survival. In contrast, the same dose of SM entrapped in liposomes prolonged the survival for all mice to more than 15 days. The therapeutic activity paralleled the dose in the liposomes, and a dose as low as 1.2 mg of SM per kg in liposomes prolonged the survival. The advantage of using liposomes was more pronounced when a larger dose of SM was employed. The liposome-entrapped drug was less toxic than the free drug. A dose of 80 mg of free SM per kg caused convulsions, but the same dose entrapped in liposomes caused no side effects. Furthermore, two doses of liposome-entrapped SM further enhanced the therapeutic effect. The efficacy of the liposome-entrapped drug was still observed in mice infected with a large inoculum of *S. enteritidis*. A tissue distribution study on SM in various organs demonstrated that liposomal SM was selectively delivered to the spleen and liver with concentrations in these organs about 100 times higher than those in mice receiving the free drug. The prolongation of survival was due to suppression of the multiplication of *S. enteritidis* as demonstrated by viable cell counts in the spleens.

Synthetic phospholipid bilayer vesicles, called liposomes, have attracted considerable interest as potent drug carriers (6). They are nontoxic and biodegradable and can protect the entrapped drugs from enzymatic attack or immune recognition until they reach the target cells. The toxicity of a drug itself can be reduced by this shielding in the liposomes. Upon intravenous injection, liposomes, especially multiple-layer vesicles, are removed rapidly by reticuloendothelial (RE) cells and at least partly localized in lysosomes, where they are slowly degraded (7, 13, 15). This behavior of liposomes has some advantages for the treatment of diseases caused by intracellular parasites (5).

In this study, experimental salmonellosis in mice was adopted as a model system of bacterial infection by intracellular parasites, and the therapeutic effect of streptomycin (SM) entrapped in liposomes was investigated. The results clearly indicated that the administration of liposome-entrapped SM could prolong the survival far more than the injection of free SM. Various applications of this method of selective drug delivery to RE cells were discussed.

MATERIALS AND METHODS

Mice. Male C57BL/6 mice were obtained from the Laboratory of Experimental Animals (Institute of Medical Science, University of Tokyo, Japan). The animals were maintained on laboratory chow and acidified chlorinated water and used when 2 to 4 months old.

Lipids. Egg yolk phosphatidylcholine was prepared by chromatography on alumina and silicic acid. Dipalmitoylphosphatidic acid and cholesterol were purchased from Sigma Chemical Co., St. Louis, Mo.

Preparation of liposomes containing SM. Liposomes were prepared from a lipid mixture of egg yolk phosphatidylcholine (5 μmol), cholesterol (5 μmol), and dipalmitoylphosphatidic acid (0.5 μmol) (negatively charged). The dried lipid film, obtained on rotary evaporation and subsequent vacuum desiccation, was dispersed in 0.5 ml of an SM solution (streptomycin sulfate, Meiji Seika Co. Ltd., Tokyo, Japan) in saline at the following concentrations: 250 mg/ml for an injection of 20 mg/kg, 125 mg/ml for 10 mg/kg, 62.5 mg/ml for 5 mg/kg, 31.2 mg/ml for 2.5 mg/kg, and 15.6 mg/ml for 1.2 mg/kg. Unencapsulated SM was removed by centrifugation at 20,000 × *g* for 20 min at 4°C, and the final pellet was resuspended in 5 ml of saline. Under these experimental conditions the entrapment of SM in the liposomes was equivalent to about 2 to 4% of the original solution.

Measurement of SM entrapped in liposomes. The amount of SM entrapped in the liposomes was determined by the method of Boxer et al. (3). Briefly, 0.5 ml of a liposome suspension was shaken with 2 ml of chloroform-methanol (2:1, vol/vol), and the upper (water) phase was recovered. A 0.25-ml portion of this water phase was evaporated and dissolved in 0.5 ml of saline. After the addition of 0.1 ml of 2 N NaOH, the solution was boiled for 3 min. Folin reagent (0.1 ml) and 0.2 ml of Na_2CO_3 were added, and the optical density was measured at 750 nm.

Evaluation of the therapeutic effect of SM in experimental salmonellosis. The virulent strain 116-54 of *Salmonella enteritidis* (10) was kindly supplied by N. Osawa (Department of Microbiology, Kitasato University School of Medicine, Sagamihara, Japan). The organisms were suspended in Trypticase-soy broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 5% skim milk plus 1% glucose and kept frozen at −80°C in small portions. For each experiment, freshly thawed bacteria from the stock solution were used. After overnight culture on Trypticase-soy agar, the organisms were suspended in saline; this

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TABLE 1. Therapeutic effect of SM entrapped in liposomes on the prolongation of survival of mice infected with *S. enteritidis*

Expt	Dosage regimen		No. of survivors/no. tested at day after challenge:											
	Agent (route)	Dose (mg/kg)	5	7	10	15	20	25	30	35	40	45	50	55
1	None	0	3/10	0.10										
	SM (i.v.)	40	6/10	0/10										
	SM (s.c.[a])	120	7/10	0/10										
	SM (s.c.)	400	10/10	4/10	0/10									
2	None	0	5/10	0/10										
	SM (i.v.)	20	5/10	0/10										
	L+SM[b] (i.v.)	20	3/10	0/10										
	L-SM[c] (i.v.)	20	10/10	10/10	10/10	9/10	5/10	4/10	4/10	3/10	3/10	2/10	2/10	0/10
3	None	0	3/10	0/10										
	L-SM (i.v.)	1.2	10/10	6/10	2/10	0/10								
	L-SM (i.v.)	2.5	10/10	10/10	6/10	0/10								
	L-SM (i.v.)	5	10/10	10/10	9/10	2/10	1/10	1/10	0/10					
	L-SM (i.v.)	10	10/10	10/10	9/10	6/10	2/10	2/10	1/10	0/10				
	L-SM (i.v.)	20	10/10	10/10	10/10	10/10	4/10	3/10	1/10	0/10				
4	None	0	6/10	0/10										
	SM (i.v.)	80	8/10	0/10										
	L-SM (i.v.)	20	10/10	10/10	10/10	9/10	5/10	2/10	2/10	1/10	0/10			
	L-SM (i.v.)	80	10/10	10/10	10/10	10/10	9/10	9/10	7/10	7/10	4/10	3/10	3/10	3/10
5	None	0	8/8	0/8										
	L-SM × 1 (i.v.)	20	8/8	8/8	8/8	7/8	4/8	3/8	1/8	0/8				
	2 × L-SM (i.v.)	20	10/10	10/10	10/10	10/10	10/10	9/10	8/10	8/10	8/10	8/10	8/10	8/10

[a] s.c., Subcutaneous.
[b] L+SM, Free SM plus empty liposomes.
[c] L-SM, Liposome-entrapped SM.

suspension, diluted to an optical density of 0.31 at 520 nm, contained approximately 3×10^8 CFU/ml. Mice were inoculated intravenously (i.v.) with 3×10^2 CFU (calculated), a dose that was 10 times the minimal lethal dose and that was uniformly lethal within 5 to 7 days. In each experiment, the number of viable cells actually injected was checked by plating on Trypticase-soy agar, and the number ranged from 2.4×10^2 to 4×10^2 CFU. Free SM or SM entrapped in liposomes was injected i.v. 24 h after inoculation, and their therapeutic effects were evaluated on the basis of the prolongation of survival.

Enumeration of *S. enteritidis* in spleens of infected mice. On various days after the administration of SM alone or SM in liposomes on day 1, three animals per group were sacrificed. Their spleens were removed and disrupted in 5 ml of distilled water with a Waring blender. Viable organisms in the spleens were enumerated by plating 0.3 ml of appropriately diluted samples on Trypticase-soy agar.

Tissue distribution studies on SM. Mice were given i.v. injections of 1 mg of SM per mouse in either the free or liposome-entrapped form. At various times thereafter, three animals of each group were sacrificed, and blood was collected by cardiac puncture. Subsequently, the spleen, liver, and kidneys were excised and homogenized in saline. After centrifugation at $1,500 \times g$, the concentration of SM in the supernatant was determined from the inhibitory activity toward the growth of *Bacillus subtilis* PCI219 (9). Estimation of SM concentrations by measurement of the zones of inhibition was kindly performed by Y. Matsuzaki and H. Morishima, Nihon Banyu Seiyaku, Tokyo, Japan.

RESULTS

Effect of free SM in experimental salmonellosis. Before starting the experiments with liposome-entrapped SM, various doses of free SM were administered to check whether free SM itself could prolong the survival of infected mice. Since the 50% lethal doses (LD_{50}s) of SM administered i.v. and subcutaneously are around 150 to 300 and 600 to 1,200

TABLE 2. Therapeutic effect of liposome-entrapped SM on infection induced with a large inoculated dose

No. of cells inoculated	Agent (mg/kg)	No. of survivors/no. tested at day after challenge:										
		4	5	7	10	15	20	25	30	35	40	45
3×10^2	None	5/5	0/5									
3×10^2	L-SM[a] (20)	10/10	10/10	10/10	10/10	9/10	6/10	5/10	5/10	3/10	1/10	0/10
3×10^4	None	3/5	0/5									
3×10^4	L-SM (20)	10/10	10/10	10/10	9/10	3/10	0/10					

[a] See Table 1, footnote *c*.

TABLE 3. Tissue distribution of SM in free or liposome-entrapped form

Agent	Tissue	Mean distribution ± SE, expressed in μg/g of organ or μg/ml of serum, at h after administration					
		1	3	6	10	20	40
SM	Serum (pooled)	9.60	0.44	0.09	<0.07	<0.07	<0.07
	Spleen	<0.89	<0.95	<0.88	<0.89	<1.18	<1.07
	Liver	0.32 ± 0.02	<0.24	0.32 ± 0.02	<0.26	<0.28	<0.28
	Kidneys	4.23 ± 0.29	1.99 ± 0.41	1.65 ± 0.59	1.15 ± 0.10	<0.79	<0.79
L-SM	Serum (pooled)	2.55	0.17	0.08	<0.07	<0.07	<0.07
	Spleen	89.5 ± 19.2	67.4 ± 9.8	56.6 ± 7.5	23.0 ± 5.3	17.4 ± 1.5	28.9 ± 8.2
	Liver	46.8 ± 1.3	28.1 ± 0.49	24.3 ± 2.1	16.4 ± 0.75	10.5 ± 0.28	4.04 ± 0.04
	Kidneys	7.01 ± 1.37	1.54 ± 0.30	0.93 ± 0.01	0.94 ± 0.08	<0.73	<0.67

mg/kg, respectively, injections of 40 mg of SM per kg were given i.v., and injections of 120 and 400 mg of SM per kg were given subcutaneously. Even with such high doses of SM, the mice survived for only 2 or 3 days longer than the untreated mice (Table 1, experiment 1).

Comparison of the therapeutic effects of liposome-entrapped and nonentrapped SM. SM was encapsulated in liposomes, and its therapeutic effect was compared with that of nonencapsulated SM. As shown in experiment 2, 20 mg of free SM per kg or the same amount of free SM plus empty liposomes could not prolong the survival of infected mice. In contrast, 20 mg of liposome-entrapped SM per kg markedly prolonged the survival. All of the mice survived for more than 15 days, and one mouse was still alive on day 54. The therapeutic activity almost paralleled the dose of SM entrapped in the liposomes as demonstrated in experiment 3, and even as small a dose as 1.2 mg of SM per kg slightly prolonged the survival.

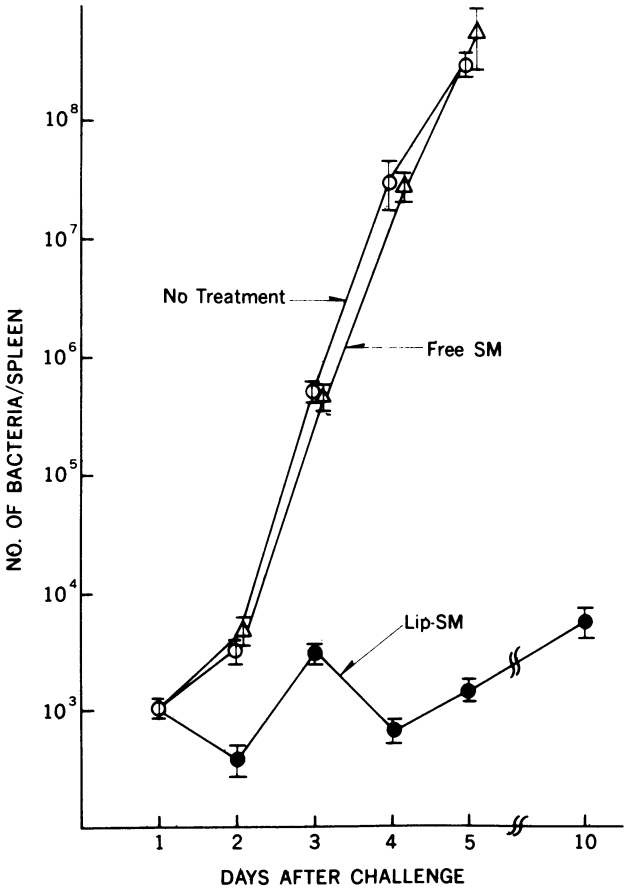

FIG. 1. Effect of SM on the multiplication of *S. enteritidis* in the spleen. Results are expressed as the means for three mice. Experimental procedures are described in the text.

Effect of a large dose of SM entrapped in liposomes. When 80 mg of free SM per kg was given i.v., all of the mice suffered convulsions. Of the 13 mice injected, one immediately fell dead. The rest of the mice recovered; 10 of them who looked healthy were selected arbitrarily as control mice and received free SM. Even in such a high dose, free SM could not prolong the survival (experiment 4). In contrast, no convulsions were observed when SM was administered in the liposome-entrapped form in a dose of 80 mg/kg. Furthermore, the prolongation of survival was more pronounced than that observed with 20 mg of liposome-entrapped SM per kg and 3 of 10 mice survived for more than 50 days.

Effect of two injections of liposomes containing SM. All of the experiments described above were done with a single injection of liposomes, and most of the mice eventually died of salmonellosis. For a complete cure, multiple injections were attempted. As the first step, the effect of two doses of liposome-entrapped SM was studied. The infected mice received liposomes containing 20 mg of SM per kg on days 1, and 6. As shown in Table 1, experiment 5, prolongation of survival was much more pronounced in the group which received two injections, and 8 out of 10 mice survived longer than 50 days.

Therapeutic effect of liposome-entrapped SM on infection induced with a large inoculated dose. The efficacy of liposome-entrapped SM was further tested in mice inoculated with a large dose of *S. enteritidis* (3×10^4). All of the mice inoculated with 3×10^4 CFU and given 20 mg of liposome-entrapped SM per kg at 24 h survived for more than 10 days (Table 2). However, all of the mice died within 20 days, and the prolongation of survival was less than that observed in mice inoculated with 3×10^2 CFU.

Tissue distribution of SM. At various times after i.v. administration of 1 mg of free SM or SM in liposomes, three mice from each group were sacrificed by cardiac puncture. The distribution of SM was studied by measuring the biological activity of tissue homogenates or serum (pooled) against the multiplication of *B. subtilis* in vitro. SM in the free form was rapidly cleared from the kidneys, and only small amounts of SM were retained in the liver and spleen

(Table 3). In contrast, when administered in the liposome-entrapped form, the concentrations of SM in the spleen and liver were more than 100 times those of free SM.

Effect of SM on the multiplication of *S. enteritidis* in the spleen. Experiments were designed to determine whether administration of SM entrapped in liposomes actually inhibited the multiplication of bacteria in the spleen. After injection of SM, free or entrapped in liposomes, in a dose of 20 mg/kg, the spleens were excised from three mice per group, and the total number of viable organisms was enumerated each day. The multiplication of bacteria was not affected significantly by administration of free SM, and the mice died between days 5 and 7 (Fig. 1). In contrast, in the mice that received liposome-entrapped SM, the multiplication of *S. enteritidis* was completely suppressed, and the number of viable cells remained at around 10^3 per spleen. The inhibition of multiplication was still observed at day 10.

DISCUSSION

In this study experimental salmonellosis, a typical disease caused by intracellular bacteria, was successfully treated with SM when the drug was delivered in a liposome-entrapped form. The therapeutic effect paralleled the dose of SM in the liposomes, and even 1.2 mg of liposome-entrapped SM per kg slightly prolonged the survival, whereas 20 mg of free SM per kg did not have any significant effect. Furthermore, the advantage of using liposomes was more evident when a large dose of SM was used; i.e., entrapment in the liposomes reduced the toxic effect and prolonged the survival much more. Two injections of SM in liposomes were found to be more efficient than a single administration. The therapeutic effect of liposome-entrapped SM was still observed in mice infected with a large inoculated dose (3×10^4 CFU).

It is clear from the SM distribution study that the therapeutic effect is at least partly due to the selective delivery of SM to RE cells. The increase in SM concentration in the spleen and liver was striking, and the levels reached more than 100 times those of free SM. Selective delivery of drugs to RE cells by liposomes has already been used for the treatment of diseases caused by intracellular parasites, especially leishmaniasis. In golden hamsters injected intracardially with *Leishmania donovani*, Alving et al. (2) found that a liposome-encapsulated antimonial drug was about 330 to 640 times more effective than the free antimonial drug in causing a drop in the death rate. New et al. (12) obtained similar results with NMR1 mice which were inoculated i.v. with the same parasite and treated with liposomal antimonial drugs. Furthermore, they demonstrated that the growth of cutaneous leishmaniasis could be reduced by i.v. administration of liposomes loaded with antimonial compounds (11). Treatment with liposome-entrapped drugs was extended to trypanosomiasis (4) and malaria (1), and significant improvement in the therapeutic effects was observed. Similarly, treatment of infectious diseases caused by intracellular bacteria can be enhanced by the use of liposomes. To our knowledge, however, there have been no papers reporting clear-cut observations, probably due to the lack of an appropriate experimental system.

There are many bacterial infections in which the main foci are RE cells, including typhoid fever, brucellosis, listeriosis, tularemia, and infection by *Pasteurella* spp. In the future, clinical application to these infectious diseases will be feasible. In brucellosis especially, daily oral administration of large doses of antibiotics over an extended period is required (for example, 2 to 3 g of tetracycline daily for several weeks). Even with such large doses of drugs, the treatment is sometimes incomplete, and administration of such doses is particularly unsuitable for older patients and patients with renal diseases. In the veterinary field, brucellosis is more common and serious. Although we have to survey more carefully the side effects of liposomes in vivo, we have not found any macroscopic side effects and have never observed sudden death on i.v. injection. In this respect, liposome therapy will be immediately applicable to brucellosis in livestock.

Preferential delivery of drugs to RE cells is also applicable to immunodeficiency of phagocytes. In chronic granulomatous disease phagocytes retain their ingestive activity, but due to the loss of their killing function, catalase-positive bacteria, especially *Staphylococcus aureus*, can multiply in the cells (8). The administration of large quantities of antibiotics cannot save the affected children from eventual death. Chronic granulomatous disease will be a good candidate for the application of liposome-entrapped drugs.

Liposomes will also be useful to augment RE cell functions. Reed et al. (14) demonstrated that macrophages activated by lymphokines encapsulated in liposomes are resistant to infection by *Leishmania donovani* subsp. *chagasi*. Thus, liposomes are attractive as a means of delivering various agents to RE cells to augment or modify their functions.

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