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Enhanced Efficacy of Liposome-Encapsulated Ribavirin Against Rift Valley Fever Virus Infection in Mice

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Administration of liposome-encapsulated ribavirin to mice led to ribavirin concentrations in the liver, the primary site of Rift Valley fever virus proliferation, that were fivefold greater than those attained with the same doses of free ribavirin. Liposomal ribavirin given at a dose of either 25 or 50 mg of drug per kg of body weight protected mice against a rapidly lethal high-titer challenge with Rift Valley fever virus, whereas similar doses of free drug or empty liposomes had no detectable benefit. Hence, tissue targeting of ribavirin with liposomes substantially increased the therapeutic index by increasing the efficacy of the treatment. By using liposomes as drug carriers, a nontoxic, low-dose regimen of ribavirin had a therapeutic effect that was comparble to that achieved with higher but potentially more toxic doses of free ribavirin.

Liposomes have been used as carriers of therapeutic agents and have been particularly successful for treating parasitic and fungal infections involving macrophages (1). For example, liposome-encapsulated antimonial drugs are in excess of 700 times more effective than nonencapsulated drugs in the treatment of experimental leishmaniasis (1, 2). In liposome-encapsulated form, amphotericin B and other antimicrobial drugs (5, 10, 12, 16, 21) and immunomodulators (9, 19) have been utilized for the treatment of microbial infections. This led us to a comparison of the chemotherapeutic efficacy of aqueous ribavirin (RIB) and liposome-encapsulated RIB (L-RIB) against Rift Valley fever virus (RVFV) infection in mice. In this infection, virus replication initially occurs within the fixed macrophages of the liver (Kupffer cells) and later in reticuloendothelial cells of other visceral organs (17).

MATERIALS AND METHODS

Drug. RIB (1-β-D-ribofuranosyl-2,4-triazole-3-carboxamide) and ^{14}C-labeled RIB (100 μCi/0.43 mg) containing ^{14}C label in the 3 position of the triazole ring (specific activity, 56.3 mCi/mol) were purchased from ICN Pharmaceuticals, Irvine, Calif.

Preparation of aqueous drug. For tissue distribution studies in normal mice, free RIB was prepared by dissolving unlabeled and ^{14}C-labeled RIB in sterile water in a ratio of 200:1 and then diluting it in saline to a final concentration of 1 mg/ml. For efficacy studies in RVFV-infected mice, free (unlabeled) RIB was dissolved in sterile saline to yield the desired dose in a volume equivalent to 1% body weight.

Liposome preparation. Lipids were purchased from the following sources: dipalmitoyl phosphatidylcholine, Sigma Chemical Co., St. Louis, Mo.; cholesterol, Calbiochem Behring, La Jolla, Calif.; and dicetyl phosphate, K & K Laboratories, Inc., Plainview, N.Y. Liposomes, prepared according to published methods (3), consisted of dipalmitoyl phosphatidylcholine-cholesterol-dicetyl phosphate in a molar ratio of 2.0:1.5:0.22.

For the encapsulated form of [^{14}C]RIB (used in tissue distribution studies in normal mice), 240 mg of cold RIB and 0.34 mg of labeled RIB were mixed, and 22 ml of the combination (75 mg/ml) was used to hydrate a dry shell of lipids obtained previously by coating the flask with 4.4 ml each of 50 mM dipalmitoyl phosphatidylcholine, 37.5 mM cholesterol, and 5.5 mM dicetyl phosphate. The final concentration of encapsulated RIB was calculated either from radioactive measurements or by determination of optical densities measured spectrophotometrically at a wavelength of 206 nm by use of a standard curve after dissolving encapsulated RIB in isopropyl alcohol. By either method, liposomes contained approximately 16 mg of drug per ml, representing a 20% encapsulation efficiency. For antiviral efficacy studies, 75 mg of cold RIB per ml was encapsulated as described above. The respective encapsulation yields of the two batches were 19 and 22%. Empty liposomes were prepared as described above, with sterile water.

Mice. Female Swiss Webster mice, 5 or 12 weeks of age, were used as specified. The mice were purchased from Harlan Sprague-Dawley, Walkersville, Md.

Virus. The Zagazig 501 strain of RVFV was isolated in Egypt during the 1977 epidemics (14). The virus was grown on Vero cells and enumerated by counting PFU under agar (14). In the efficacy studies, challenge doses of 250 or 5,000 PFU were employed as indicated.

RESULTS

Distribution of [^{14}C]RIB, with or without liposomes, in normal mouse tissue. Groups of 12 mice, 12 weeks of age, were injected intravenously with 12.5, 25, or 50 mg of free RIB or L-[^{14}C]RIB per kg. At 1, 24, and 48 h later, four mice from each group were sacrificed by CO_2 asphyxiation and exsanguinated. The livers, lungs, and spleens were removed, weighed, and frozen for storage. The liver (divided into two parts), lung, and spleen samples were placed on absorbent pads, and their $^{14}CO_2$ contents were collected in a Packard 306 Tri-Carb tissue oxidizer. The $^{14}CO_2$ trapped in the samples was counted in a Searle model 6880 Mark III liquid scintillation counter with Permaflour V1 scintillation mixture.

At 60 min after the administration of L-[^{14}C]RIB to

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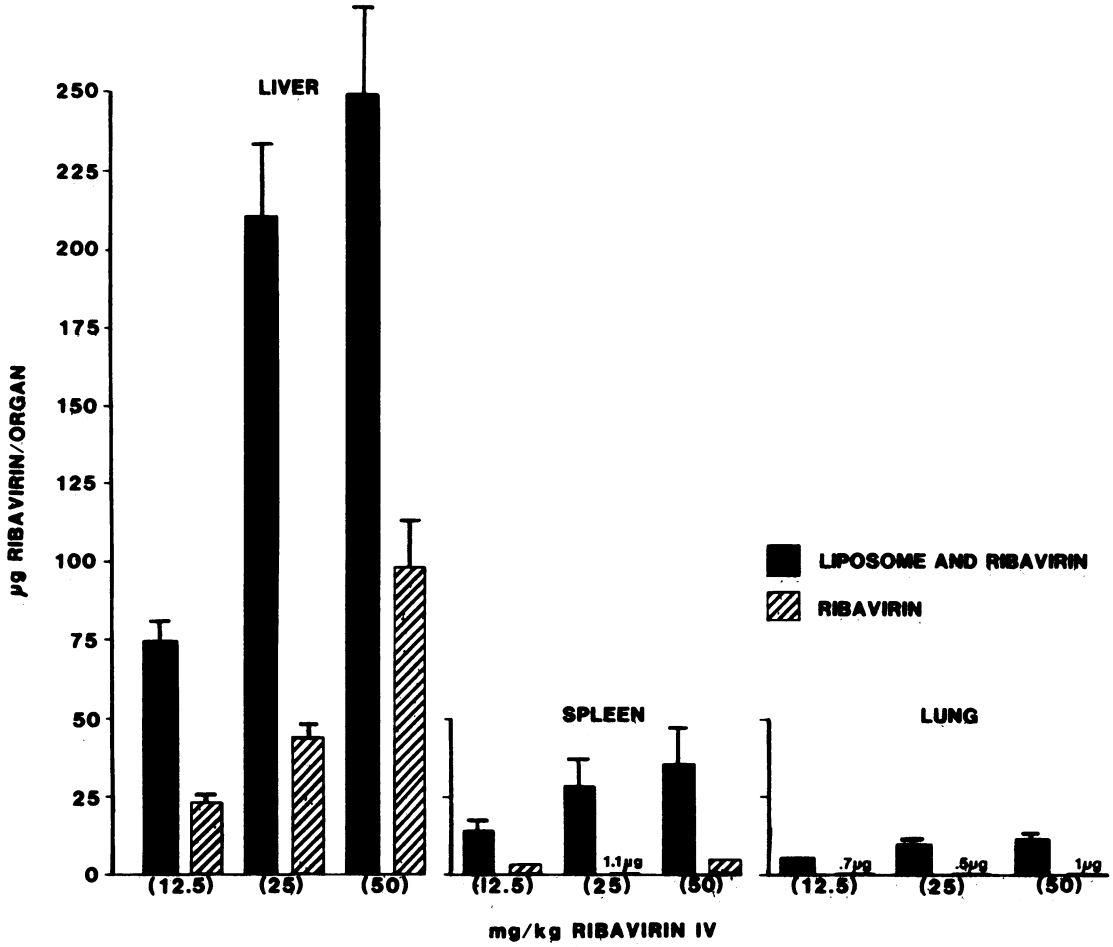

FIG. 1. Distribution of [^{14}C]RIB, with or without liposomes, in mouse tissue 1 h after injection. IV, Intravenous.

noninfected mice, the amount of drug in the liver (74, 210, and 250 μg) was 2.5- to 5-fold higher than that obtained with corresponding doses of free [^{14}C]RIB: 22, 43, and 98 μg per organ (Fig. 1). The amount of RIB in the liver increased in a dose-related fashion, although an apparent plateau was approached at a L-RIB dose of 50 mg/kg. The concentration of the liposome-delivered drug in spleen was lower than that in the liver (13.0, 27.0, and 34.0 μg per organ) but was 7- to 24-fold greater than that attained with unencapsulated drug (2.0, 1.1, and 3.8 μg per organ) and was also dose dependent. The levels of radioactivity in the lung after administration of free [^{14}C]RIB did not exceed background levels, the equivalent of 0.5 μg. In contrast, concentrations of 5.0, 8.0, and 10 μg were present in this organ after administration of L-[^{14}C]RIB. At 1 h after injection of 12.5, 25, and 50 mg of L-[^{14}C]RIB per kg in the three major target organs, 29.9, 39.9, and 31.7% of the drug, respectively, was recovered, in contrast with the respective recoveries of 9.4, 7.3, and 11.1% of the injected aqueous [^{14}C]RIB, representing 3.2-, 5.4-, and 2.3-fold dose-related increases, respectively, in the proportional distribution of this drug in the organs assayed. In all three tissues, RIB levels decreased below background levels after 24 h, regardless of the mode of delivery.

Efficacy of L-RIB or RIB against low-titer dose of RVFV. Five-week-old Swiss Webster mice were inoculated subcutaneously with 250 PFU of RVFV and were treated intravenously on days −1, 0 (within 60 min after inoculation), 1, 2, and 3 with either 10 mg of L-RIB or 10, 25, or 50 mg of RIB per kg, saline, or empty liposomes containing 1.5 μmol of phospholipid.

Treatment with a 10-mg/kg dose of L-RIB resulted in a survival rate, 20 days after infection, that was fivefold higher than that achieved with a 10-mg/kg dose of liposome-free RIB (Fig. 2). This low-dose (10-mg/kg) regimen of L-RIB resulted in a survival rate after 12 days that was equivalent to that achieved in experimental groups receiving 25 and 50 mg of RIB per kg. Because of the short treatment schedule, the therapeutic effect was reduced when examined beyond day 12. Treatment with empty drug-free liposomes (equivalent to the amount injected with 50 mg of L-RIB per kg) was not effective.

Efficacy of longer treatment against a higher dose of RVFV. Mice were inoculated with 5,000 PFU of RVFV and treated over a course of 11 days (Fig. 3). Treatment, consisting of doses of 12.5, 25, or 50 mg of L-RIB, RIB, saline, or empty liposomes per kg, was initiated within 60 min after inoculation and spaced as indicated by the arrows in Fig. 3. RIB was significantly less effective against the higher dose of 5,000 PFU than against 250 PFU, even though the treatment was administered for an extended schedule (up to 11 days). Because of the increased total drug load, 12-week-old mice were employed in the study, as they have a better tolerance than younger mice for larger amounts of RIB. The high RVFV dose resulted in the death of all untreated mice by

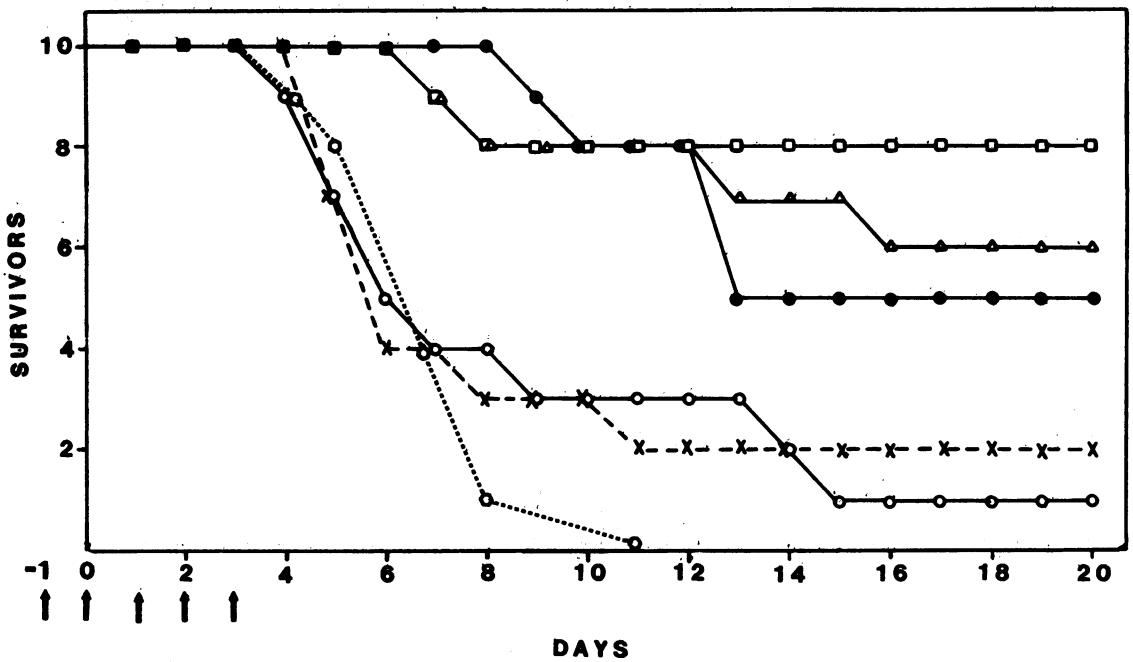

FIG. 2. Efficacy of L-RIB, aqueous RIB, or empty liposomes (L) against RVFV infection (day 0) in 5-week-old female Swiss Webster mice. Arrows indicate the days of treatment. Symbols: □, RIB at 25 mg/kg; △, RIB at 50 mg/kg; ●, L-RIB at 10 mg/kg; ×, saline, ○, RIB at 10 mg/kg; ⬡, liposomes.

day 4. Furthermore, 29 of 30 mice injected with 12.5, 25, or 50 mg of RIB per kg were dead by day 11. Even though the treatment with aqueous drug affected the survival time relative to the saline control, the increase was not statistically significant ($P > 0.01$). In contrast, therapeutic regimens of 12.5, 25, or 50 mg of L-RIB per kg resulted in survival of 18 of 30 animals (60%) by day 11 and 14 of 30 animals (47%) by day 50 ($P < 0.01$ relative to saline control; $P < 0.0001$ relative to RIB treatment). Even the lowest dose (12.5 mg/kg) resulted in more long-term survivors than the highest

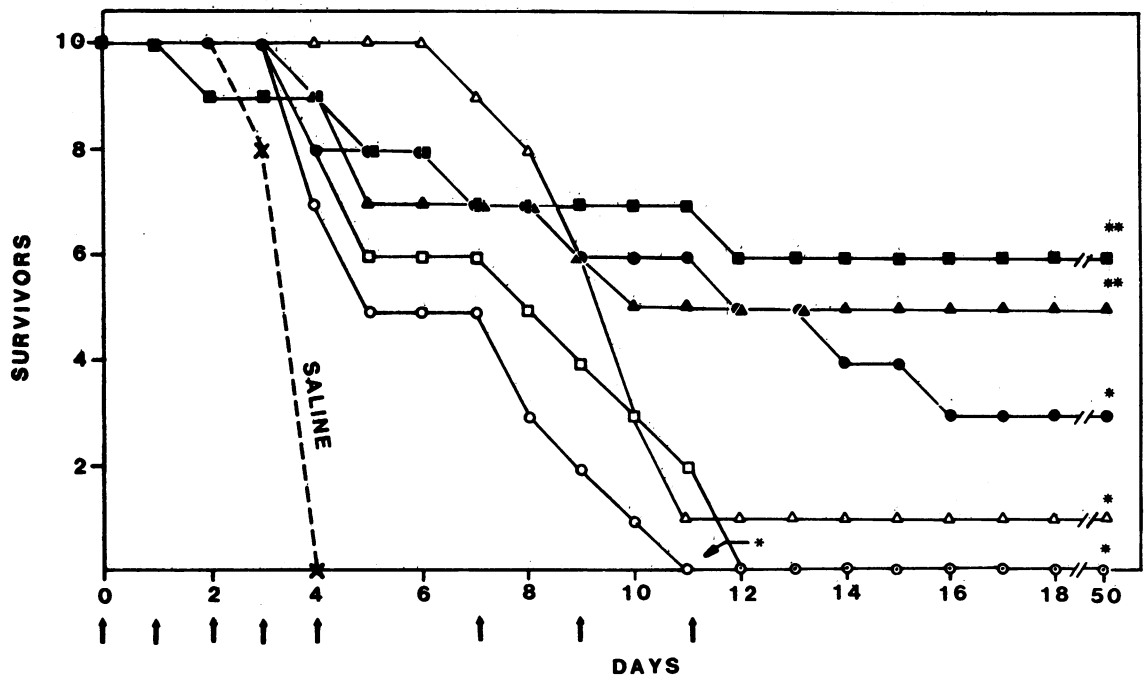

FIG. 3. Efficacy of L-RIB or RIB against RVFV infection (day 0) in 12-week-old female Swiss Webster mice. P values were calculated by the Fisher one-tailed exact test. *, $P > 0.01$ (not significant compared with untreated mice); **, $P \leq 0.01$ (significant compared with untreated mice). The P value, by the Fisher one-tailed exact test, of all L-RIB-treated mice versus RIB-treatment was 0.0001. Arrows indicate the days of treatment. Symbols: ■, L-RIB at 25 mg/kg; ▲, L-RIB at 50 mg/kg; ●, L-RIB at 12.5 mg/kg; △, RIB at 50 mg/kg; ⊙, RIB at 25 mg/kg; ○, RIB at 12.5 mg/kg; ×, saline.

concentration of free drug (50 mg/kg). There was no substantial difference in the efficacy of the two highest doses of L-RIB, suggesting the occurrence of a plateau similar to that observed for the uptake and distribution of L-[^{14}C]RIB in liver, lung, and spleen (Fig. 1). Therapy with empty liposomes containing 1.5 μmol of phospholipid, an amount equivalent to that delivered with 50 mg of L-RIB per kg, had no effect beyond the saline controls (data not shown).

DISCUSSION

RVFV infection has long been a major epizootic pathogen in sub-Saharan Africa (14). In humans, this agent typically causes self-limiting febrile illness, and approximately 1% of the reported cases progress to fatal hemorrhagic fever or encephalitis (7). Experimental RVFV infection in mice results in fatal hepatitis or encephalitis in virtually all animals (6, 17). High doses of RIB have been used successfully to treat experimental RVFV infections in several strains of mice (11, 20). Treatment failures occur at low drug doses, however, resulting in death due to either hepatitis or, a few days later, encephalitis.

This report describes the first successful in vivo use of a liposome-encapsulated compound for the treatment of a lethal virus-induced infection in mice. The rationale for this approach was based on the known propensity for liposomes to be phagocytosed by macrophages of the liver, spleen, and lung (1, 2, 22), where RVFV is known to replicate (18).

In the present study, we demonstrated that L-RIB had a high level of efficacy against a high dose of RVFV under conditions in which the same dose of free drug was completely ineffective in terms of long-term survivors. The efficacy of a low dose of L-RIB equalled that of a fivefold-higher dose of the aqueous drug when the amount of the virus challenge was much lower. This therefore demonstrates that RVFV infection in mice can be accessible to treatment by liposomal drug carriers.

With respect to tissue distribution, liposomal delivery of RIB substantially increased the levels obtained in liver, spleen, and lung 1 h after injection. Proportionally, fivefold more drug was distributed into the three major target organs by L-RIB than by aqueous RIB. A previously published study in rodents has demonstrated that a peak drug concentration occurs at 30 min in tissues (8). In that study, the RIB concentration was already slightly diminished after 1 h, and RIB had completely disappeared from the tissues at 24 h (8).

The liposome-mediated increase in RIB concentration in the spleen is of particular importance, since the virus spreads from the liver into the reticuloendothelial cells of the spleen (17). The amount of RIB in the spleen is negligible after injection of free drug. With liposomal delivery, the quantity of RIB in the spleen increased by 7- to 24-fold and equalled the RIB concentration in the liver on the basis of weight. The presence of higher amounts of RIB in the spleen, therefore, may account for some of the increased antiviral efficacy.

The attainment of greater levels of antiviral agent in tissue by use of a biodegradable phospholipid delivery system offers substantial potential chemotherapeutic advantages. Therapeutic efficacy was achieved with a lower dose of compound, as low as 25 mg/kg, which is nontoxic and not otherwise effective against a large challenge without the carrier. On the basis of our extensive experience with aqueous RIB, intermittent doses from days 5 to 11 gives results identical to those achieved with daily treatment. Any additional treatment has no beneficial effect, since deaths beyond day 7 are due to involvement of the central nervous system (about 10 to 20% of total deaths). Although RVFV infection is fatal in mice within 4 to 6 days, additional experiments are now under way to further explore the therapeutic efficacy of L-RIB during the narrow window (1 to 2 days) that exists between infection and overwhelming lethal disease.

Effects of RIB on RVFV titers in vivo were not examined in this study. According to our experience, 25 PFU of RVFV was sufficient to cause death in 80 to 100% of the mice, with a high level of circulating virus in serum at the time of death (15), and theoretically at least one virus particle will kill the host. It is therefore assumed that the presence of a higher amount of RIB in the target organs (as was the case with L-RIB in normal mice) might be translated to increased antiviral activity in vivo. The biological activity of RIB has been reviewed extensively (4, 11), but it is not known whether such activity has been altered by liposome-mediated drug targeting.

In humans, oral regimens of 600 mg of RIB per day (ca. 10 mg/kg of body weight) for 28 consecutive days are well tolerated (4). Dosages exceeding 1,200 mg of RIB per day for 14 days may be, albeit rarely, associated with reversible reductions in hemoglobin, hematocrit, and total erythrocyte number. In humans, life-threatening viral infections caused by Lassa fever virus have been treated with large doses of RIB (30 g over 10 days) (13). The hemotoxicity associated with such high-dose regimens of RIB therapy of Lassa fever virus might be obviated by the use of liposome-mediated targeting to the macrophages of the liver to enhance the efficacy of a low-dose regimen. Current therapy for a number of potentially lethal bunyavirus, flavivirus, and arenavirus infections, including Lassa fever, relies on the availability of immune sera of sufficiently high titers from convalescent patients. Such therapy, however, is seriously impeded by the scarcity of antiserum. Since Lassa fever and other human viral pathogens of the bunyavirus, flavivirus, and arenavirus families replicate in phagocytic cells and the major drug-related toxicity occurs outside of phagocytic cells, it is reasonable to expect that liposomes might increase drug efficacy without inducing toxicity. Hence, the use of liposomes given either prophylactically or a short time after exposure to the virus offers a potential approach for the treatment of these life-threatening viral infections.

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