Enhanced Iron Removal From Liver Parenchymal Cells in Experimental Iron Overload: Liposome Encapsulation of HBED and Phenobarbital Administration


The effectiveness of N,N’-bis[2-hydroxybenzyl]-ethylenediamine-N,N’-diacetic acid (HBED) in removing radioiron introduced into the parenchymal cells of mouse liver as \(^{59}\)Fe-ferritin has been investigated. The effectiveness of HBED, an iron chelator of low water solubility, has also been compared with that of desferrioxamine (DF), an iron chelator of high water solubility and currently in clinical use for treatment of transfusional iron overload. Using the \(^{59}\)Fe excretion as the measure of effectiveness of chelation therapy and a standardized single chelator dose of 25 mg/kg, we have found that: (1) a saline suspension of HBED, prepared by sonication and given intraperitoneally to mice, promotes a small but significant increase in excretion of radioiron compared to the untreated controls, whereas DF, in its free form, is ineffective; (2) HBED encapsulated in lipid bilayers of liposomes and given intravenously is superior to nonencapsulated HBED; (3) DF encapsulated in small unilamellar liposomes is ineffective in removing iron given in the form of ferritin; (4) administration of phenobarbital in drinking water, at a concentration of 1 g/liter, induces a 30%-55% increase of iron excretion from untreated control mice and also from mice given HBED either in liposome-encapsulated or nonencapsulated form. We have demonstrated that HBED is superior to DF for removal of storage iron from liver parenchymal cells and that liposomes are useful carriers for iron chelators of low water solubility.

MATERIALS AND METHODS

Materials

Cholesterol and synthetic dipalmitoyl phosphatidylcholine were obtained from Sigma Chemical Company, St. Louis, MO; stearylamine was from K.K. Laboratories, Plainview, NY; desferrioxamine (Desferal) from Ciba Pharmaceutical Company, Summit, NJ; radioactive iron in the form of \(^{59}\)FeCl\(_3\), in 0.1 M HCl (1 mCi/ml) from Amersham Corp., Arlington Heights, IL; and sodium phenobarbital from J.J. Baker Chemical Company, Phillipsburg, NJ. HBED was kindly provided by Dr. C.G. Pitt (Research Triangle Institute, NC). Reagent grade chemicals and “spectranalyzed” organic solvents were used in all preparations.

Preparation of Liposomes With HBED Incorporated in the Lipid Bilayers and DF in the Aqueous Compartments

(A) Multilamellar liposomes (ML) containing HBED were prepared by mixing dipalmitoyl phosphatidylcholine, cholesterol, and stearylamine in 5–10 ml of chloroform at a molar ratio of 3.3:2:3.1. HBED (20–40 mg) was dissolved in 2–5 ml of methanol and added to the above lipid mixture. The chloroform and methanol mixture was dried to an evenly spread thin film in the bottom of a round flask using a rotary evaporator. Saline solution was then added to the flask, under constant stirring, with the temperature controlled at 50°C with a water bath. The resulting multilamellar liposomes, designated ML-Dipalm-HBED, were filtered successively through Nuclepore filters of 2, 1, and 0.6 μm in pore size. Liposomes of about 0.6 μm and smaller would be present in this ML preparation. They were then washed twice with saline before use. During the washing

From the Division of Biological and Medical Research, Argonne National Laboratory, Argonne, IL.

Supported in part by Grant AM 21592 from the National Institutes of Health.

Submitted May 11, 1982; accepted February 1, 1983.

Address reprint requests to Dr. Y. E. Rahman, Division of Biological and Medical Research, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, IL 60439.

0006-4971/83/6106-0030$01.00/0


209
step by centrifugation, the majority (presumably not all) of the liposomes smaller than 0.1 μm were discarded along with the supernatant.

(B) Unilamellar liposomes (UL) containing DF or HBED were prepared by a 30-min sonication of the corresponding ML preparations, as described earlier. Because UL containing DF were more effective than the corresponding ML in removing storage iron from liver parenchymal cells, only unilamellar DF liposomes were used in this study.

(C) The amount of iron chelator encapsulated within liposomes was determined by the addition of a small tracer amount of 59Fe to the solution of HBED or DF for liposome preparation. The dosage of encapsulated chelator was then determined by the 59Fe-radioactivity found in the final suspension of liposomes.

Determination of Size and Stability of Liposomes Containing HBED or DF

The size of multilamellar liposomes was limited to the pore size (0.6 μm) of the last of the Nuclepore filters used. The size of unilamellar liposomes was determined by electron microscopy after negative staining. The stability of liposomes was determined by the rate of drug leakage, using a dialysis method described previously. The effect of blood plasma on the leakage of HBED or DF from liposomes was also tested.

Preparation of 59Fe-Labeled Soluble Ferritin

59Fe-labeled ferritin was prepared according to Bjorklid and Helgeland. Iron dextran (Imferon) was given subcutaneously, in a volume of 0.1 ml containing 5 mg of iron, every other day for 3 injections. After the third Imferon injection, three injections of 59FeCl3 (10 μCi/mouse) in 1% sodium citrate were given intraperitoneally at 1, 6, and 24 hr to each mouse. Radioactive ferritin was isolated from livers of 6–10 mice 1 day after the last 59Fe citrate injection and was purified. Iron content of the labeled ferritin was determined according to Drysdale and Munro.

Chelation Therapy Experiments for Removal of Parenchymal Cell Iron

Female CF1 mice, 2–4 mo of age and weighing 25–32 g, were used. At 24 hr before chelation therapy, each received a single intravenous injection of 0.2 ml of 59Fe-ferritin, ranging from 60,000 to 75,000 cpm, containing 100 μg iron. (This represents about 45% of the mouse liver burden of iron.) Twenty-four hours later, about 90% of the 59Fe-ferritin was recovered in the liver, and of this, over 90% was in the parenchymal cells; only a negligible amount of radioiron was excreted during these 24 hr. Thus, injection of 59Fe-ferritin introduces the iron specifically into the liver parenchymal cells and provides a suitable model system for iron loading in this cell compartment.

The chelators HBED and DF, either in liposome-encapsulated or in nonencapsulated free form, were administered in 0.2 ml saline as a single intravenous injection at a dose of 25 mg/kg. The one exception was that nonencapsulated HBED was injected intraperitoneally to avoid complications following intravenous injections of small particulates. Before injection, it was suspended in saline and sonicated for 10 min. For all therapy experiments, the HBED suspensions and liposome-encapsulated HBED or DF were freshly prepared. All mice were killed 7 days after the injection of the chelator.

Preparation of Excreta for Analysis of 59Fe-Radioactivity

For chelation therapy experiments, mice were housed in stainless steel cages, five per cage, with plastic-backed absorbent paper below the bottom grid of the cage. Pooled samples of urine and of feces from each group were collected on the paper for 59Fe-radioactivity measurements in a Beckman Gamma 800 automatic counting system.

Phenobarbital Administration

Various experimental groups of mice were given sodium phenobarbital in their drinking water (1 g/liter), starting immediately after the injection of HBED or DF in various forms. Phenobarbital remained in the drinking water until the mice were killed on day 7 after chelation therapy.

RESULTS

Characterization of Liposomes Containing HBED or DF

The incorporation of HBED in multilamellar liposomes ranged from 15% to 20% (based on the known amount in the original drug solution), and 10%–12% was found in unilamellar liposomes. The incorporation of DF in UL was 5%–10%.

The UL-Dipalm-HBED liposomes were limited in size by the last Nuclepore filter used, 0.6 μm in diameter. With this filtering method, liposomes of 0.6 μm in diameter and less would be collected as ML liposomes. However, during each centrifugation step, care was taken to remove the majority of the small liposomes (<0.1 μm) that remained in the supernatant.

Unilamellar liposomes, containing either HBED or DF, have a diameter of ~0.07 μm as measured by electron microscopy after negative staining.

Liposomes containing HBED and DF were quite stable; less than 10% of the encapsulated iron chelator...
Table 1. ANOVA

<table>
<thead>
<tr>
<th>Source</th>
<th>Prop</th>
<th>df</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment</td>
<td>.012</td>
<td>2</td>
<td>41.54</td>
</tr>
<tr>
<td>Treatment</td>
<td>.368</td>
<td>2</td>
<td>1329.18</td>
</tr>
<tr>
<td>Day</td>
<td>.586</td>
<td>6</td>
<td>704.76</td>
</tr>
<tr>
<td>Exp x Trt</td>
<td>.014</td>
<td>4</td>
<td>26.01</td>
</tr>
<tr>
<td>Trt x Day</td>
<td>.015</td>
<td>12</td>
<td>9.25</td>
</tr>
<tr>
<td>Error</td>
<td></td>
<td></td>
<td>1.000</td>
</tr>
</tbody>
</table>

Exp, experiment; TRT, treatment; Prop, proportion of the total sums of squares; df, degrees of freedom; F, the calculated statistic from the analysis.

Fig. 2. Daily excretion in urine plus feces of $^{59}$Fe from mice given various treatments once, 24 hr after injection of $^{59}$Fe-ferritin. All values are means derived from 5 mice. Symbols and drug doses are the same as in Fig. 1.

leaked out after 1 hr incubation in the presence of undiluted mouse plasma.

Iron Excretion From Mice Given $^{59}$Fe-Ferritin

Figures 1 and 2 show the results of a representative experiment. The radioiron excretion from mice given $^{59}$Fe-ferritin and treated 24 hr later with a single dose of HBED or DF (25 mg/kg) in various forms is presented.

When mice are given ferritin, the major pathway of iron excretion is via the bile and feces. Thus, the iron excreted was almost entirely in the feces (Fig. 1).

The results presented in Figs. 1 and 2 show the following effects on iron excretion of treatment of iron overload achieved by ferritin administration. (1) Treatment with DF, either in nonencapsulated or in liposome-encapsulated form, is ineffective. (2) HBED, suspended in saline and injected intraperitoneally, promotes a moderate increase in iron excretion compared with untreated controls. (3) HBED encapsulated either in ML or UL is better than its nonencapsulated form. (4) Iron excretion promoted by nonencapsulated HBED is limited to the 24 hr after treatment. In contrast, although the effect of liposome-encapsulated HBED is highest at day 1 after chelation therapy, an increased excretion can still be observed up to the third day after treatment (Fig. 2).

Statistical Analysis

In order to assess the repeatability of the chelating effect by various treatments, results from three independent replications of the experiment were collected into a single data set. An analysis of variance (Table 1) was conducted with percent injected $^{59}$Fe-radioactivity as the response variable. The overall fit of the model was highly significant ($p < 0.0001$), with an $R^2$ of 0.995. Treatment differences [UL-Dipalm-HBED, free HBED, free DF (UL-Dipalm DF or controls)] accounted for approximately 37% of the variability in the data, while differences between the three independent replications of the experiment accounted for only 1% of the variability measured as the proportion of the total sums of squares (Table 1). The repeatability of
the experiments can be readily seen by the clustering of identical treatments between experiments and within days (Fig. 3).

The large F-value associated with the treatment effect (Table 1) suggested a Duncan's multiple range test, using the mean squared error from the analysis of variance. Significant differences ($p < 0.0001$) were found between all treatments within days. A similar analysis conducted on experiments demonstrated a similarity of experiments within treatments (Table 2).

**Effect of Phenobarbital on Iron Excretion From Mice Given $^{59}$Fe-Ferritin**

Administration of phenobarbital in the drinking water significantly increases the amount of iron excreted by control mice and mice treated with HBED in encapsulated or free form (Fig. 3). The increased excretion ranges from about 30% to 55%. The enhancement of iron excretion by phenobarbital continues throughout the experimental period of 7 days, as illustrated in Fig. 4 for the groups of mice receiving nonencapsulated HBED treatment.

**DISCUSSION**

The most significant contribution of the present study is the demonstration that an iron chelator of low water solubility, i.e., HBED, can be effectively delivered into animals by incorporating it in the lipid bilayers of liposomes. Also significant is the finding that HBED encapsulated in suitable liposomes can enhance iron excretion from mice given ferritin, a compound selectively placing iron in the liver parenchymal cells.\(^{16,17}\) Serious toxic manifestations in patients suffering from chronic iron overload are usually attributed to excess iron in the liver parenchymal compartment, rather than to the reticuloendothelial compartment of liver.\(^{18,19}\) HBED should, therefore, be particularly useful for treatment of patients with significant iron loading in the liver parenchymal cells.

In this study, HBED delivered in small unilamellar liposomes was not significantly more effective than that delivered in large multilamellar liposomes in promoting iron excretion from mice given ferritin. This finding is somewhat surprising, given the fact that the spaces between the endothelial fenestrae lining the liver sinusoids have a diameter of about 0.1 $\mu$m;\(^{20}\) only liposomes smaller than 0.1 $\mu$m, such as the UL used here, should therefore be expected to pass these fenestrae and reach the liver parenchymal cells. The multilamellar liposomes used here were larger, about 0.6 $\mu$m, and only a small proportion (i.e., the proportion of

**Table 2. Summary of Duncan’s Multiple Range Comparisons of Treatments**

<table>
<thead>
<tr>
<th>Day</th>
<th>C</th>
<th>L</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NS</td>
<td>AC, BC</td>
<td>AB, AC</td>
</tr>
<tr>
<td>2</td>
<td>NS</td>
<td>AC, BC</td>
<td>AC, B</td>
</tr>
<tr>
<td>3</td>
<td>NS</td>
<td>A, BC</td>
<td>AC, B</td>
</tr>
<tr>
<td>4</td>
<td>NS</td>
<td>A, BC</td>
<td>AC, B</td>
</tr>
<tr>
<td>5</td>
<td>NS</td>
<td>A, BC</td>
<td>A, BC</td>
</tr>
<tr>
<td>6</td>
<td>AC, BC</td>
<td>A, BC</td>
<td>A, BC</td>
</tr>
<tr>
<td>7</td>
<td>NS</td>
<td>A, BC</td>
<td>A, BC</td>
</tr>
</tbody>
</table>

C, free DF (UL-Dipalm-DF or saline controls); L, UL-Dipalm-HBED; F, free HBED; between experiments (A, B, C) within days (1, 2, 3, 4, 5, 6, 7). Group letters indicate similar experiments. NS indicates all experiments were similar.
liposomes smaller than 0.1 μm that still remained in the population after the washing centrifugation step) would be expected to be able to pass through the endothelial fenestrae to the parenchymal cells.

Phenobarbital has been reported to stimulate synthesis of hepatic microsomal haem protein, and this increased haem synthesis appears to be associated with a mobilization of intrahepatic storage iron. Our present results show that phenobarbital can indeed mobilize the storage ferritin from the mouse liver and enhance iron excretion in feces. Although such findings are interesting, elucidation of the mechanisms underlying this stimulated iron mobilization needs further investigation. Also, the practical usefulness of phenobarbital in chelation therapy is yet to be demonstrated.

In recent years, various biologic systems have been introduced as carriers for drug delivery. For iron chelation therapy, Green and coworkers encapsulated DF in red blood cell ghosts and showed that the system was superior to the continuous DF infusion method introduced by Propper et al. Green et al. also reported that DF in red cell ghosts enhanced iron excretion from the reticuloendothelial storage compared to the amount excreted after treatment with free DF; however, no improvement was observed for iron excretion from the parenchymal storage. This observation may be explained by the fact that red blood cells are too large to pass the endothelial fenestrae of the liver sinusoids. Encapsulation of DF in liposomes also significantly increased their effectiveness in removing storage iron from the reticuloendothelial system of the liver. A further advantage of the use of liposomes is that they can be prepared in different ways (e.g., varying in size and surface properties) to confer suitable properties for specific delivery of the iron chelators to the liver parenchymal cells.

ACKNOWLEDGMENT

We thank Dr. C. G. Pitt, Research Triangle Institute, Research Triangle Park, North Carolina, for providing us with the HBED to carry out the studies presented in this paper.

REFERENCES

15. Bjorklid E, Helgeland L: Sex difference in the ferritin content of rat liver. Biochim Biophys Acta 221:583, 1970
Enhanced iron removal from liver parenchymal cells in experimental iron overload: liposome encapsulation of HBED and phenobarbital administration

YE Rahman, EA Cerny, EH Lau and BA Carnes