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EVALUATION OF THE SCHISTOSOMICIDAL EFFICACY OF LIPOSOME - ENTRAPPED OXAMNIQUINE

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SUMMARY

Oxamniquine (OXA) was sucessfully encapsulated in small unilamellar vesicles using a pH gradient method. This procedure led to a high drug encapsulation efficiency (> 85%) at a drug to lipid molar ratio of 1/10. Moreover, these liposomes were found to retain encapsulated OXA efficciently under dialysis conditions at 37° C. Liposome-entrapped OXA (LOXA), OXA, and empty liposomes were tested against *Schistosoma mansoni* in a murine model. LOXA produced a significant reduction of the worm burden compared to the other preparations, when inoculated by subcutaneous route (s.c.) with 10 mg OXA/kg animal one day before the infection, and 3, 7, and 14 days after. However, LOXA was not effective when given 7 days before, or 35 days after infections. OXA, in the free form, was effective in relation to the untreated group, only when administered 3 days after the infection. Maximum effect of LOXA, with 97% reduction of the parasite number, was observed when the preparation was given s.c.one day before the infection. On the other hand, LOXA inoculated intraperitoneally one day before the infection didn't show any reduction of the parasite count. It can be concluded that LOXA is more effective than OXA for the treatment of experimental schistosomiasis, particularly when administered subcutaneously at a time close to the infection.

KEY WORDS: *Schistosoma mansoni*; Schistosomiasis; Liposome; Oxamniquine.

INTRODUCTION

The only drugs currently available for the treatment of Manson's schistosomiasis are oxamniquine (OXA) and praziquantel. A first limitation of these drugs is their low effectiveness in the treatment of acute toxaemic schistosomiasis [11], that has been related to their low activity against immature *S. mansoni* [16]. Another important limitation is the occurrence of drug resistance and drug tolerance [3,5]. In this context, the development of new drugs and the improvement of available drugs are of great importance.

Recently, liposome-entrapped praziquantel was shown to be schistosomicidal in experimental mice, when administered 2 weeks before infection [1]. Such prophylactic activity was not observed when the drug was administered in the free form. The beneficial effect of liposomes was attributed to their targeting properties to the liver and their slow drug-release properties.

The objective of the present work was to evaluate the schistosomicidal efficacy of liposome-entrapped oxamniquine (LOXA) in a murine *S. mansoni* model. The influence of factors, such as the time of treatment and the route of inoculation, was investigated. The comparative study of LOXA and OXA efficacies allowed us to evaluate, to some extent, modification of the pharmacokinetic/biodistribution of OXA could affect its anti-schistosomal activity.

MATERIALS AND METHODS

Parasite and host

Cercariae (LE strain of *Schistosoma mansoni*) shed by laboratory-reared and infected *Biomphalaria glabrata* were used throughout the study. Swiss albino male mice, weighing about 20 g, were infected by subcutaneous route with about 60 or 90 cercariae of *S. mansoni.*

Encapsulation of OXA in liposomes

Oxamniquine (OXA-Pfizer LTD., England) was encapsulated in liposomes, using a transmembrane pH gradient procedure, previously described [2, 12] and modified as follows. Small unilamellar vesicles were formed by ultrasonication (Lab-Line Ultratip Labsonic systems) of a 80 mM L-α-

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distearoylphosphatidylcholine (DSPC - Sigma Chemical Co., St. Louis, U.S.A.) dispersion in a 0.3 M sodium citrate pH 2 buffer. A transmembrane pH gradient was then created by adjusting the extraliposomal pH to 7 with a 0.4 M NaOH, 0.033 M Na_2HPO_4 solution. OXA was then added to the resulting liposome suspension (DSPC concentration = 30 mM) at a final concentration of 3 mM. Finally, the drug-liposome mixture was incubated for 5 min at 56° C, which resulted in OXA encapsulation. Empty liposomes were prepared using the same procedure, but in the abssence of drug.

Evaluation of the efficiency and stability of OXA encapsulation

The encapsulation efficiency of OXA in liposomes, and the ability of liposomes to retain encapsulated OXA, were evaluated under dialysis conditions. The drug-liposome mixture (1 ml at 1 mM OXA) was introduced in a dialysis tubing (cellulose esters membrane with MWCO 1,000), and then dialysed at 37°C against 250 ml PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.2). A solution of OXA in PBS (1 ml at 1 mM OXA) was used in parallel to get information about the time-course of release of the free drug. Aliquots of both preparations (50 µl) were taken at different times over a 6-hour period, mixed with ethanol (950 µl) to disrupt the liposomes, and finally filtered (0.22 µm) to eliminate lipid aggregates. OXA concentration was determined, using OXA maximum UV absorption at 245 nm. Results were expressed as the percentage of remaining OXA in the dialysis tubing as a function of time.

Treatment of animals and recovery of worms

The first set of experiments, was performed in order to evaluate the influence of time of treatment on the activity of OXA, LOXA and empty liposomes when given by the subcutaneous route. Animals were treated with a single dose, before (7 or 1 day) or after (3, 7, 14 or 35 days) infections.

The second set of experiments, was performed in order to compare the efficacy of the subcutaneous to that intraperitoneal route. In this case, animals were treated with a single dose one day before infection.

In each experiment, four groups of 10 mice were analysed. The first group (LOXA) was treated with 10 mg of liposome-entrapped OXA / kg body weight and a second group (OXA) was inoculated with a solution of OXA in PBS (10 mg OXA / kg body weight). The third group (LEMP) was given empty liposomes (at the same lipid dose as that given to LOXA group), and the last group (CONT) remained without treatment.

Ten mice of each group were sacrificed by cervical fracture, 42 days after infection, and the adult worms were recovered by perfusion using the PELLEGRINO & SIQUEIRA[15] technique with minor modifications, and counted under a dissecting microscope, to evaluate the worm burden and the trematode distribution within the portal system.

The effectiveness of LOXA and that of OXA were determined by calculating the percentage of reduction of worms in LOXA and OXA groups in relation to LEMP and CONT groups, respectively, according to GONNERT &ANDREWS [7] with minor modifications. So the worm burden reduction rate was calculated according to the formulas below:

% of worm reduction in OXA group = 100 x (mean worm count in CONT group - mean worm count in OXA group) / mean worm count in CONT group

% of worm reduction in LOXA group = 100 x (mean worm count in LEMP group - mean worm count in LOXA group) / mean worm count in LEMP group

When the difference between a treated group (LOXA or OXA) and its respective control group (LEMP or CONT) was not statistically significant, the reduction was considered as "NS" (not-significant).

Statistical methods

Statistical comparisons were done using Student's *t* test and analysis of variance, and significance of $p < 0.05$.

RESULTS

Encapsulation efficiency and stability

Fig. 1 displays the kinetics of OXA release under dialysis conditions at 37°C, when OXA was introduced as a solution or as the drug-liposome mixture. After 6 hours of incubation, more than 95% of original free OXA was lost, whereas only 14% of OXA initially present in the OXA-liposome mixture was released. This data indicates that more than 85% of OXA was initially associated with liposomes in the OXA-liposome mixture, and that associated OXA was efficiently retained by liposomes. We also observed that LOXA was still present at the site of inoculation, as a yellow milky deposit, even one week after administration (data not shown).

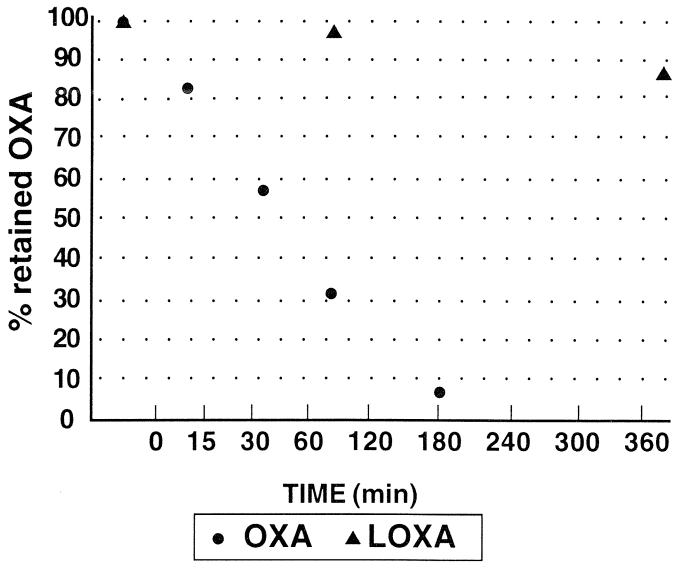

Fig. 1 - Percentage of retained oxamniquine in a dialysis tubing as a function of time when introduced as an oxamniquine solution (OXA) or as a suspension of oxamniquine-containing liposomes (LOXA).

Schistosomicidal efficacy of LOXA

Table 1 displays the mean worm counts in mice groups, 42 days after infection. Each evaluation included two treated groups, one with OXA and the other with LOXA (10 mg OXA / kg body weight), and two control groups, one inoculated with empty liposomes (LEMP) and other untreated (CONT). In this first set of experiments, all the treated groups were inoculated by subcutaneous route. The comparison between LEMP and CONT shows that empty liposomes did not induce any reduction of parasite count. On the other hand, LOXA produced a significant reduction of worm burden compared to the other preparations, when inoculated one day before, and 3, 7, and 14 days after infections. However, LOXA was not effective, when given 7 days before, or 35 days after infections. OXA, in the free form, was effective compared to the control, only when administered 3 days after infection. Maximum effect of LOXA, with 97% reduction of parasite count, was observed when the preparation was given subcutaneously one day before the infection. It is also noteworthy that this was the only group, among all treated groups, to display granuloma-free animals (8 out of 10 mice) and worm-free animals (5 out of 10 mice).

In a second set of experiments, the efficacy of the intraperitoneal treatment was compared to that of the subcutaneous one (table 2). Our data shows that LOXA was ineffective in reducing the worm burden, when inoculated intraperitoneally one day before the infection.

DISCUSSION

In this study, OXA was successfully encapsulated in liposomes. The high OXA encapsulation efficiency (> 85%) at a drug to lipid molar ratio of 1/10 is consistent with previous observations that amphipatic weak base compounds strongly accumulate in liposomes in response to transmembrane pH gradients [12].

The superiority of LOXA over OXA in reducing parasite count was demonstrated following subcutaneous injection, one day before, and 3, 7, and 14 days after infections. This effect is related to the ability of liposomes to modify *in vivo* the fate of encapsulated drugs. At least three different mechanisms may account for the enhanced drug efficacy.

First, the ability of liposomes to slowly release the encapsulated drug and to protect it from rapid metabolism and elimination, may lead to lower but more prolonged blood levels, which in turn, as previously proposed [6], may result in a higher drug activity.

The second mechanism is related to the ability of liposomes to target the liver, where the parasites are located 2-3 weeks after infection. This mechanism was recently proposed [1], to explain the higher schistosomicidal activity of subcutaneously-injected liposome-entrapped praziquantel as compared to free praziquantel. It has been also suggested that liposomes might be

TABLE 1

Anti-schistosomal activity of liposome-entrapped oxamniquine (LOXA), oxamniquine (OXA) and empty liposomes (LEMP) when given subcutaneously, 7 and one day before, and 3, 7, 14 and 35 days after infection.

TIME OF TREATMENT	MEAN WORM COUNT IN MICE GROUP				% REDUCTION	
	LOXA	OXA	LEMP	CONT	LOXA	OXA
- 7 days +	32.4 ± 9.8	30.7 ± 10.8	31.3 ± 9.7	28.2 ± 8.6	NS	NS
- 1 days *	2.3 ± 3.9	62.5 ± 20.1	76.8 ± 13.3	64.1 ± 18.6	97%	NS
+ 3 days *	31.1 ± 38.2	65.5 ± 19.5	78.3 ± 17.6	87.3 ± 15.6	60.3%	25%
+ 7 days +	20.8 ± 9.8	25.0 ± 12.7	39.8 ± 12.3	30.9 ± 14.6	47.7%	NS
+ 14 days +	29.2 ± 8.2	36.5 ± 6.3	40.1 ± 11.9	42.8 ± 10.6	27.2%	NS
+ 35 days *	72.8 ± 26.6	58.8 ± 20.0	71.8 ± 29.1	71.1 ± 38.0	NS	NS

+ animals were infected with 60 cercariae on day 0
* animals were infected with 90 cercariae on day 0
NS = Not significant

TABLE 2

Anti-schistosomal activity of liposome-entrapped oxamniquine (LOXA), oxamniquine (OXA) and empty liposomes (LEMP) when given by subcutaneous (S.C.) and intraperitoneal (I.P.) routes one day before the infection.

ROUTE OF INOCULATION	MEAN WORM COUNT IN MICE GROUP				% REDUCTION	
	LOXA	OXA	LEMP	CONT	LOXA	OXA
S.C *	2.3 ± 3.9	62.5 ± 20.1	76.8 ± 13.3	64.1 ± 18.6	97%	NS
I.P. +	39.3 ± 8.5	36.0 ± 8.5	39.3 ± 11.9	39.9 ± 8.6	NS	NS

+ animals were infected with 60 cercariae on day 0
* animals were infected with 90 cercariae on day 0
NS = Not significant

directly ingested by the *Schistosoma* parasites due to their high affinity for phospholipids [13]. In our case, however, this mechanism may account for only part of LOXA activity, as intraperitoneal injections, which increase liposome targeting to the liver [10], did not produce any reduction of the parasite number.

Thirdly, the ability of liposomes to create a deposit at the subcutaneous inoculation site, may increase locally OXA concentration, affecting the parasites during the adaptation and migration process in the vertebrate host [14]. In fact the same occurs with the glucocorticoids which have their effects on worm burden only if they are administered around the time of infection [4, 8, 9]. So this effect may reflect the early death of larvae in the treated animals before or after reaching the lungs. This mechanism could explain the greater activity of the liposome preparation, when administered at a time close to the infection, and is supported by the fact that treatment by the intraperitoneal route did not produce any reduction of the worm burden.

In conclusion, LOXA is more effective than OXA for the treatment of experimental schistosomiasis, particularly when administered by subcutaneous route at a time close to the infection.

Our data suggests that any carrier system, capable or slowly releasing OXA at the subcutaneous level, should be effective in the prevention of schistosomiasis.

RESUMO

Avaliação da eficácia esquistossomicida da oxamniquina encapsulada em lipossomas.

Oxamniquina (OXA) foi encapsulada em vesículas unilamelares pequenas de distearoilfosfatidilcolina usando-se técnica de encapsulação ativa em gradiante de pH. Este procedimento produziu uma alta eficiência de encapsulação (> 85%) com uma razão molar de 1/10, além de reter, eficientemente, a droga encapsulada sob condições de diálise à 37° C.

OXA encapsulada (LOXA), OXA livre (OXA), (10 mg/kg respectivamente) ou lipossomas vazios foram testados durante o curso da infecção experimental pelo *Schistosoma mansoni.*

Verificou-se uma redução significativa do número de parasitos recuperados no grupo tratado com LOXA, por via subcutânea 1 dia antes ou 3, 7, ou 14 dias após a infecção, quando comparado aos grupos controle, lipossomas vazios e OXA. LOXA não foi efetiva quando administrada 7 dias antes ou 35 dias após a infecção. OXA livre (OXA) apresentou atividade em relação ao grupo controle quando administrada 3 dias após a infecção. O efeito máximo de LOXA, com 97% de redução da carga parasitária, foi observado quando administrada um dia antes da infecção.

Por outro lado, não foi observada redução do número de parasitos quando LOXA foi inoculada intraperitonealmente 1 dia antes da infecção.

Pode-se concluir que LOXA é mais efetiva que OXA no tratamento da esquistossomose experimental quando administrada subcutaneamente em período próximo à infecção.

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