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Therapeutic effect of chloroquine(CQ)-containing immunoliposomes in rats infected with *Plasmodium berghei* parasitized mouse red blood cells: comparison with combinations of antibodies and CQ or liposomal CQ

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The potential therapeutic application of chloroquine-containing immunoliposomes (Fab′-lipCQ) in a *Plasmodium berghei* malaria model was studied. Extending a previously described in vivo model (Peeters et al. (1988) Biochim. Biophys. Acta 943, 137–147) it was demonstrated that injection of antimouse red blood cell (anti-mRBC) Fab′-lipCQ was significantly more effective than liposome-encapsulated chloroquine (lipCQ) or free chloroquine in delaying or preventing a patent infection after intravenous injection of parasitized mouse red blood cells (p-mRBC) in rats. The results could be improved by injecting synchronized infected cells instead of non-synchronous p-mRBC in order to minimize the presence of free parasites which could easily infect rat RBC. It was further demonstrated that sequential injection of anti-mRBC IgG and lipCQ or chloroquine resulted in complete inactivation of the injected parasitized cells while Fab′-lipCQ administration resulted in a maximum score of 50% at an equal chloroquine, protein and phospholipid dose. In this report the potential of the concept of drug targeting for the effective treatment of a disease, which manifests in blood cells, was demonstrated.

Introduction

One of the approaches to achieve site-specific drug delivery is the application of immunoliposomes (antibody – or antibody fragments – usually covalently linked to liposomes). Extensive reviews on the preparation of immunoliposomes and 'in vitro' studies with these immunoliposomes are available [1–7]. The 'in

Abbreviations:

chol	cholesterol
CQ	chloroquine
Cr-mRBC	^{51}Cr-labelled mouse red blood cell(s)
DTT	dithiothreitol
Fab′-lip	Fab′-liposome(s)
Fab′-lipCQ	chloroquine-containing Fab′-liposome(s)
FCS	fetal calf serum
lipCQ	chloroquine-containing liposomes
MPB-PE	maleimidophenylbutyrate-phosphatidylethanolamine
MPS	mononuclear phagocyte system
mRBC	mous red blood cell(s)
mRETS	mouse reticulocytes
P.	*Plasmodium*
PBS	phosphate-buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
p-mRBC	parasitized mouse red blood cell(s)
p-mRETS	parasitized mouse reticulocytes
PL	phospholipid(s)
PS	phosphatidylserine
RBC	red blood cell(s)
RETS	reticulocytes
REV	reverse-phase evaporation vesicle(s)
REV-Fab′	reverse-phase evaporation vesicle(s) bearing Fab′
RPMI 1640	Roswell Park Memorial Institute medium, number 1640
SMPB	succinimidylmaleimido-4-(*p*-phenylbutyrate)
syn-p-mRETS	in vitro synchronized parasitized mouse reticulocytes

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vivo' application of immunoliposomes for targeted delivery of therapeutic or diagnostic agents has been the subject of investigation in a limited number of studies (reviewed in Ref. 8). From these studies, the anatomical and physiological constraints interfering with successful site specific delivery of drugs with immunoliposomes can be derived. In principle, all cells and tissues directly in contact with blood are potential candidate structures for targeting with immunoliposomes after intravenous (i.v.) administration. Blood cells (long circulation times) are good candidates to study the interaction between immunoliposomes and the appropriate target cell 'in vivo'. In a recent study [9] it was observed that a high level of i.v. injected target cells (mouse red blood cells; mRBC) was maintained in the blood compartment of untreated rats for several hours. Rapid elimination of these target cells (mRBC) from the blood compartment, however, was observed after i.v. injection of specific (anti-mRBC) immunoliposomes or free antibody (fragments).

In the present study this model system was used to demonstrate functional drug targeting with chloroquine (CQ)-containing anti-mRBC immunoliposomes to *Plasmodium berghei* (*P. berghei*) infected mRBC. The therapeutic response after injection of CQ-laden immunoliposomes was compared with the response after administration (i.v.) of free specific anti-mRBC IgG and CQ-containing liposomes (lipCQ; without a specific homing device) or free CQ. The therapeutic efficacy of treatment with CQ-containing immunoliposomes was significantly better than that of lipCQ or free CQ treatment at the same CQ dose level or free anti-mRBC IgG. In addition, it was found that sequential application of IgG/lipCQ or IgG/CQ combinations was more effective than the CQ-containing immunoliposomes in preventing the development of a patent infection in rats.

Materials and Methods

Chemicals. Phosphatidylcholine (PC), phosphatidylserine (PS), dithiothreitol (DTT), cholesterol (chol) and pepsin were obtained from Sigma Chemicals (St. Louis, MO, U.S.A.). Phosphatidylethanolamine (PE) was purchased from Lipid Products (Nutfield, U.K.). Succinimidylmaleimido-4-(*p*-phenylbutyrate) (SMPB) was purchased from Pierce Chemical Company (Rockford, U.S.A.). Chloroquine diphosphate met the requirements of the British Pharmacopoeia. Protein A Sepharose CL-4B and other Sephadex gels were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). SE-23 was obtained from Servacell (Heidelberg, F.R.G.) and silica gel (70–325 mesh) from Merck (Darmstadt, F.R.G.). $Na_2{}^{51}CrO_4$ was obtained from Amersham International (Amersham, U.K.). All reagents were of analytical grade.

Animals. Outbred male Swiss mice (6 weeks old, ±25 g) and male Wistar rats (50–70 g; young rats were used because of their relative high sensitivity to the infection) were obtained form colonies of the animal facility of the University of Nijmegen. They were kept in plastic cages and recieved standard food (RMH, Hope Farms) and water ad libitum.

Parasite. Plasmodium berghei (strain K173) was maintained by weekly intraperitoneal (i.p.) inoculation of 10^5 p-mRBC from infected donor mice into healthy mice of the same strain and sex. Parasitemia was determined from blood smears, made from tail blood stained with May Grünwald-Giemsa's solution.

Purification of mRBC and p-mRBC. Normal mRBC suspensions were prepared by passing heparinized (5 U/ml) whole blood through a column containing three volumes of Sephadex G-150 Superfine and one volume of the ion exchanger SE-23 to remove white blood cells and platelets [10]. p-mRBC were isolated and purified essentially as described before [10]. Briefly, infected blood was taken from mice at day 7 after infection and heparin (5 U/ml) was added to prevent clotting. Blood was diluted once with medium (RPMI 1640 (Gibco) buffered with 20 mM Hepes and 10 mM sodium carbonate and supplemented with 10% fetal calf serum (FCS)). White blood cells and platelets were removed as described for mRBC. The cells recovered in the column eluate were diluted to five times the original blood volume and layered on top of a Percoll (Pharmacia) cushion with a density of 1.094. After centrifugation (1500 × *g*, 20 min) the interphase was harvested and washed three times with medium. The cells were counted and kept on ice until they were injected. Microscopic analysis revealed that the suspensions contained over 90% p-mRBC of all developmental stages (from early trophozoite to mature schizont). To remove merozoites the p-mRBC suspensions were washed (1000 × *g*, 5 min) twice immediately before injection.

Synchronization of infection. Synchronization was performed according to the methods described by Mons et al. [11] and Janse et al. [12] with minor modifications. Infected mouse reticulocytes (p-mRETS) used for synchronization were obtained from two Swiss mice made anaemic by bleeding from the retro-orbital plexus (200–250 μl of blood) one day before and one day after infection with 10^5 p-mRBC obtained from a passage mouse on day 7 after infection. Five days after infection, the parasitemia was 8–10% with 60% of the parasites in reticulocytes (p-mRETS). Blood of the infected animals was taken by cardiac puncture under sterile conditions, supplemented with heparin (5 U/ml) and diluted in complete medium (RPMI 1640, buffered with 20 mM Hepes and 10 mM sodium hydrogencarbonate and supplemented with 10% FCS, 2 mM L-glutamine and penicilline (100 U/ml)). After centrifugation (1500 × *g*, 5 min) the buffy coat was removed, the pelleted

cells washed with complete medium and diluted with normal mRBC (1 : 1, obtained in a similar way) in a final volume of 50 ml. This suspension was cultivated in an erlenmeyer flask (500 ml) in a shaking apparatus at 37 °C in an atmosphere of 85% nitrogen, 10% oxygen and 5% carbon dioxide. After 17 h of cultivation the majority of the parasites had matured to the schizont stage (microscopic analysis). The synchronized p-mRETS (syn-p-mRETS) were separated by density centrifugation (1600 × *g*, 20 min) on a cushion of Nycodenz (55% v/v). The interphase was collected and washed with complete medium. The cells were counted and stored on ice. $5 \cdot 10^7$ of these mature schizonts were mixed with $(2-3) \cdot 10^8$ mRETS and immediately injected (i.v.) in a normocytic mouse (the erythropoiesis of this mouse was inhibited by the i.p. injection of 0.5 ml heparinized normal whole blood 7 and 4 days previously). The mRETS that were mixed with the syn-p-mRETS were obtained from mice that were hyperbled from the retro-orbital plexus (200–250 μl) 5 and 2 days previously. These mRETS were isolated by density centrifugation on a Percoll cushion as described above. 17 h after inoculation of syn-p-mRETS and mRETS, blood was taken and the syn-p-mRETS were isolated as described above. Microscopic analysis revealed that over 90% of the parasites were in the trophozoite stage and in reticulocytes (syn-p-mRETS). These syn-p-mRETS were stored on ice until they were injected.

Preparation of CQ-containing immunoliposomes. Maleimido-4-(*p*-phenylbutyrate)-phosphatidylethanolamine (MPB-PE) was prepared as described earlier [13]. MPB-PE bearing reverse-phase evaporation vesicles were prepared by the method of Szoka and Papahadjopoulos [14] with minor modifications [9,15]. The lipid composition was chol/PC/PS/MPB-PE 10 : 9.5 : 1 : 0.5. The lipids were dissolved in 4 volumes of diethyl ether (freshly distilled) in a round bottom flask. After addition of glass beads, one volume of CQ solution (225 mg CQ-diphosphate = 139 mg CQ base per ml in 100 mM Tris buffer, final pH 4.0) was added and the two phases were emulsified by sonication for 5 min and subsequently mixed on a vortex mixer for one min. Diethyl ether was removed under reduced pressure in a nitrogen atmosphere with a rotary evaporator at 20 °C. The remaining liposome dispersion was subsequently extruded through 0.4 and 0.2 μm polycarbonate membranes (Nuclepore Corp., Pleasanton, U.S.A.). After ultracentrifugal sedimentation (to remove non-encapsulated CQ) at 80 000 × *g* during 45 min (three times) no free CQ was detectable in the supernatant immediately after these washing steps. The final MPB-PE bearing lipCQ pellet was suspended in isotonic 100 mM sodium acetate buffer, pH 7.4 (with sodium chloride) and used immediately for coupling to freshly prepared anti-mRBC Fab′-fragments (prepared from anti-mRBC rabbit IgG) as described earlier [9,15]. Fab′-liposomes (Fab′-lip) with either specific anti-mRBC Fab′ or control Fab′ (prepared from a feline leukemia virus (FeLV) monoclonal IgG_1) with (Fab′-lipCQ) or without encapsulated CQ were prepared in a similar way. In order to further study the effect of the liposomal Fab′ density on clearance and organ distribution, immunoliposomes were prepared with lower ratios, e.g., 15.3, 5.5 and 1.5 μg/μmol phospholipid by lowering the Fab′ concentration in the coupling reaction medium to 0.25, 0.1 and 0.03 mg/ml, respectively.

CQ determination. Total amount of CQ (expressed as CQ-base in liposome suspensions) was determined spectrophotometrically at 341 nm, pH 1. Free CQ (CQ leakage) was determined spectrofluorimetrically at pH 9.3, excitation and emission wavelength 330 nm and 383 nm, respectively, and expressed as percentage of the total CQ content as described earlier [16].

Distribution studies. The method used to study the clearance of ^{51}Cr labelled mRBC (Cr-mRBC) has been described elsewhere [9]. The effect of the density of specific Fab′ coupled to liposomes on the clearance of i.v. injected ^{51}Cr labelled mRBC was studied using immunoliposomes with different Fab′/phospholipid ratios injected in a constant dose of 10 μmol phospholipid per rat. The subsequent distribution of the Cr-mRBC was determined as described earlier [9]. Total recovery was approximated by summarizing the radioactivity found in blood, spleen, liver and lungs. Each data point is the mean ± S.D. from the data of four animals.

Therapeutic studies. 10^5 freshly isolated p-mRBC or syn-p-mRETS were i.v. injected into the tail vein of rats in a total volume of 0.2 ml. Anti-mRBC Fab′-lipCQ, control Fab′-lipCQ, lipCQ or free CQ (in a dose of 0.8 mg CQ per rat unless otherwise indicated), anti-mRBC Fab′-lip or buffer was i.v. injected in a total volume of 0.2 ml into the tail vein 10 min later. In cases where the combination of anti-mRBC IgG/lipCQ or anti-mRBC IgG/CQ were tested, the two components were injected separately 10 min after injection p-mRBC or syn-p-mRETS. The dose of anti-mRBC IgG was 140 μg per rat (unless otherwise indicated) which was equal (on protein basis) to the amount of Fab′-lipCQ (25 ± 5 μg Fab′/μmol phospholipid, injected dose 5 μmol per rat). Parasitemia and mortality were recorded. Parasitemia was determined daily by counting 10^4 mRBC in May Grünwald-Giemsa's stained blood smears.

In previous experiments it was found that at equal protein concentration the capacity to agglutinate mRBC was better for Fab′-lip preparations than for $F(ab')_2$ [15] and IgG (results not shown).

In separate experiments, using an RBC agglutination assay [15] it was demonstrated that anti-mRBC IgG, $F(ab')_2$, Fab′-lip and Fab′-lipCQ reacted equally well with p-mRBC and syn-p-mRETS (results not shown).

Other methods used. Lipid phosphate was determined by the colorimetric method of Fiske and SubbaRow [17]. Protein was determined by the method of Peterson [18]. The purity of the phospholipids and synthesized MPB-PE was analysed by the TLC method described by Heath et al. [19].

Results

Characterization of Fab'-lipCQ

Analysis of 12 independently prepared batches of immunoliposomes to be tested in therapy exhibited an encapsulation capacity of 183 ± 31 μg CQ per μmol phospholipid and a mean particle size of 0.32 ± 0.05 μm. The amount of covalently coupled Fab' in the standard procedure was 25 ± 5 μg per μmol phospholipid. No leakage of CQ or change in particle size was observed after 6 months of storage under nitrogen at 4°C (results not shown). Nevertheless, fresh preparations were used in all animal experiments.

Clearance and organ distribution of Cr-mRBC in rats after injection with Fab'-lip: effect of Fab' density on liposomes

In a previous study [9] the Fab' density on liposomes did not critically influence opsonization and subsequent elimination of Cr-mRBC by these immunoliposomes from the blood stream of rats. Thus, ratios of 30.6 and 11.6 μg Fab'/μmol phospholipid resulted in similar clearance curves and organ distributions. In order to further study the effect of the liposomal density of Fab' molecules on clearance and organ distribution, immunoliposomes were prepared with lower ratios, e.g., 15.3, 5.5 and 1.5 μg/μmol phospholipid. This was

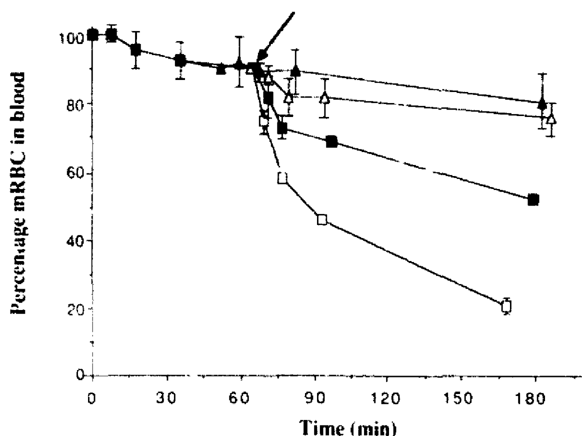

Fig. 1. Effect of liposomal Fab' densities; 1.5 (△), 5.5 (■) and 15.3 (□) μg Fab'/μmol phospholipid on the blood level of Cr-mRBC ($1.5 \cdot 10^9$) in rats after i.v. administration of Fab'-lip. As control, bare liposomes were injected (▲). In all cases a total amount of 10 μmol phospholipid was injected in a total volume of 0.5 ml, 1 h after injection of Cr-mRBC (arrow). Each data point represents the mean ± S.D. of four animals. Small S.D. values are not shown.

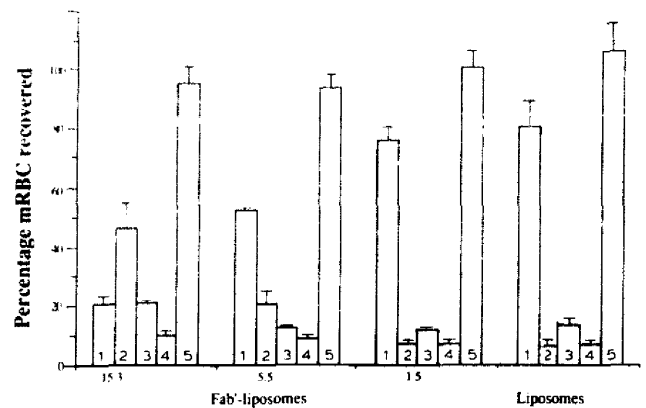

Fig. 2. Recovery of Cr-mRBC in blood (1), spleen (2), liver (3), lungs (4) and their sum (5). Animals were killed, 2 h after i.v. administration of liposomes or anti-mRBC Fab'-lip with different liposomal Fab'-densities (indicated as μg Fab'/μmol phospholipid). Presented data are the means ± S.D. from four animals. For further details see legend of Fig. 1.

achieved by lowering the Fab' concentration in the coupling reaction medium to 0.25, 0.1 and 0.03 mg/ml, respectively. The antigen binding capacity of the Fab'-lip, determined by hemagglutination assay [15], and expressed as the hemagglutination titer (HA) was 2^9, 2^5 and 2^3, respectively. Control immunoliposomes (anti-FeLV Fab'-lip) did not agglutinate mRBC under similar conditions. The results of experiments with these immunoliposomes and control preparations are presented in Figs. 1 and 2. The clearance curves and tissue distributions obtained after treatment with liposomes or control immunoliposomes (anti-FeLV Fab'-lip; results not shown) were essentially the same as for specific (anti-mRBC) Fab'-lip with the lowest ratio (1.5 μg Fab'/μmol phospholipid) (Figs. 1 and 2). Increasing the anti-mRBC Fab' density resulted in a faster elimination of Cr-mRBC (Fig. 1) and enhanced localization of the ^{51}Cr label in the liver and particularly in the spleen (Fig. 2).

Effect of Fab'-lipCQ administration on intravenous infection of rats with non-synchronous parasitized mRBC

To study the therapeutic effect of the immunoliposomes in the above described model, p-mRBC were injected i.v. in rats. The effect of an i.v. injection of specific anti-mRBC Fab'-lipCQ on the development of patency in rats infected with 10^5 p-mRBC was compared with that of control Fab'-lipCQ, lipCQ, free CQ or buffer in three independent experiments. The results are summarized in Table 1. When buffer was injected, parasitemia became patent 4 to 5 days after infection; a similar result was obtained when anti-mRBC Fab'-lip (without encapsulated CQ) was administered. Administration of control Fab'-lipCQ, lipCQ or free CQ all delayed patency significantly and to the same extent.

TABLE I

Effect of Fab′-lipCQ administration on the development of patency in rats after intravenous infection with non-synchronous parasitized mRBC

10^5 p-mRBC were i.v. injected followed 10 min later by the indicated treatment (i.v.). CQ was given in a dose of 0.8 mg per rat. Patency is defined as at least 1 parasitized rat RBC per 10^4 rat RBC.

Treatment	Number of rats used	Mean day of patency (P) ± S.D.
anti-mRBC Fab′-lipCQ	12	9.6 ± 0.5
LipCQ	9	6.4 ± 0.5
CQ	9	6.2 ± 0.4
Control Fab′-lipCQ	6	6.3 ± 0.5
anti-mRBC Fab′-lip	6	4.4 ± 0.5
Buffer	9	4.2 ± 0.4

Treatment with specific anti-mRBC Fab′-lipCQ further delayed patency significantly when compared to the effect after control Fab′-lipCQ, lipCQ or free CQ administration.

In an additional experiment the number of p-mRBC per rat was varied. To determine the effect of the 'infection load', rats received 10^4, 10^5 or 10^6 p-mRBC followed by the same therapeutic regimens: anti-mRBC Fab′-lipCQ, lipCQ or free CQ. The results with respect to the delay of patency were essentially the same after injection of 10^4 or 10^6 p-mRBC when compared to those obtained after infection with 10^5 p-mRBC (Table I). In all experiments the rats eventually died from the infection within 27 days.

Thus, despite the fact that anti-mRBC Fab′-lip can eliminate the mRBC from the blood, this can not prevent the normal development of the infection. CQ in any form delays patency and better so in combination with anti-mRBC Fab′-lip (Fab′-lipCQ).

Effect of administration of IgG / lipCQ or IgG / CQ combinations on intravenous infection of rats with non-synchronous parasitized mRBC

In previous experiments [9] it was also possible to eliminate mRBC efficiently from the circulation of rats by administration of anti-mRBC IgG. In the experiments described here this possibility was exploited in combination with CQ treatment. When treatment was successful the rats developed no infections and were scored as parasite negative. In case rats developed patency, the infection was eventually fatal within 26 days after infection. The combined results of two experiments using the same experimental set up as described above but testing anti-mRBC IgG, in combination with lipCQ or CQ, instead of anti-mRBC Fab′-lipCQ are described in Table II. The results show that administration of anti-mRBC IgG alone was not sufficient to prevent the development of a patent infection (results were equal to buffer administration; patency became detectable 4–5 days after infection for both treatments). The delay in patency as observed for lipCQ or CQ treatment described in Table I, was confirmed. In addition, a high proportion of the rats was radically cured when anti-mRBC IgG was administered combined with lipCQ or CQ, a phenomenon not observed after treatment with anti-mRBC Fab′-lipCQ (Table I).

When the dose of anti-mRBC IgG in the combined IgG/lipCQ or IgG/CQ treatments was reduced from 140 to 70 μg per rat only 4 out of 8 rats infected with 10^5 p-mRBC did not develop patency (for both regimens). This reduced therapeutic efficiency parallels the reduced clearance efficiency observed in rats injected with mRBC and subsequently treated with different doses of anti-mRBC IgG as demonstrated previously [9].

Effect of administration of Fab′-lipCQ, IgG / lipCQ or IgG / CQ on intravenous infection of rats with synchronized parasitized mRETS

The above described experiments were performed in rats infected with non-synchronous p-mRBC. The IgG and Fab′-fragments used in these experiments are directed against mRBC membranes and are supposed to be non-reactive to parasite molecules. Since one parasite is able to infect a mouse or rat (results not shown) the presence of one free merozoite in the preparation before or during the experiment can cause infection. Although p-mRBC preparations were washed before administration to remove merozoites, rupture of schizonts and release of merozoites after injection can limit the therapeutic efficiency. Thus, experiments were carried out with syn-p-mRETS to demonstrate that the presence of merozoites may have interfered with therapeutically effective drug targeting in the above described experi-

TABLE II

Effect of treatment with IgG/lipCQ or IgG/CQ combinations on the development of patency in rats after intravenous infection with non-synchronous parasitized mRBC

Treatment	Mean day of patency ± S.D. of positive rats	Number of rats radically cured [a]
anti-mRBC IgG/lipCQ [b]	13	8 (9)
anti-mRBC IgG/CQ	– [c]	6 (6)
CQ	6.2 ± 0.4	0 (6)
LipCQ	6.3 ± 0.5	0 (6)
anti-mRBC IgG	4.7 ± 0.5	0 (6)
Buffer	4.2 ± 0.5	0 (6)

[a] No parasites were detected during a 28 days period in rats infected with 10^5 p-mRBC (i.v.). Total number of rats used in two independent experiments is given in parentheses.

[b] anti-mRBC IgG dose was 140 μg per rat (in 0.2 ml buffer) equivalent to the amount of Fab′ (138 μg) of anti-mRBC Fab′-lipCQ. The dose of CQ was 0.8 mg per rat.

[c] In the treated group no positive animal was obtained because all the treated animals were radically cured.

TABLE III

Effect of treatment with Fab'-lipCQ on the development of patency in rats after intravenous infection with synchronized parasitized mRETS

Treatment	Mean day of patency ± S.D. of positive rats	Number of rats radically cured [a]
anti-mRBC Fab'-lipCQ [b]	10.0 ± 1.7	6 (12)
lipCQ	6.8 ± 0.5	0 (8)
CQ	6.5 ± 0.5	0 (8)
Buffer	5.2 ± 0.5	0 (8)

[a] No parasites were detected during a 28 days period in rats infected with 10^5 syn-p-mRETS (i.v.).

[b] CQ dose was 0.8 mg per rat. The data are summarized from two independently performed experiments.

ments. It was therefore expected that the therapeutic efficacy would be higher with synchronized cells. Syn-p-mRETS used in subsequent experiments exhibited > 90% trophozoites and no schizonts or merozoites (microscopic analysis). The results of two independent experiments using the syn-p-mRETS are summarized in Table III and can be compared to those described in Table I. These results indicate that the therapeutic efficacy of anti-mRBC Fab'-lipCQ was improved when treatment was given to rats infected with syn-p-mRETS. The synergistic effect of antibody (anti-mRBC Fab') and CQ in the anti-mRBC Fab'-lipCQ preparations already observed in the experiments described in Table I was confirmed (Table III).

Finally the therapeutic efficacy of anti-mRBC Fab'-lipCQ was compared to the efficacy of IgG/lipCQ and IgG/CQ in rats infected with 10^5 syn-p-mRETS using a CQ dose of 0.6 mg (instead of 0.8 mg) CQ per rat. The dose of CQ was reduced to differentiate between the therapeutic potential of IgG/CQ and IgG/lipCQ. The results of this experiment are described in Table IV. The data confirm an improved therapeutic efficacy of anti-mRBC Fab'-lipCQ when rats infected with syn-p-mRETS were treated, even at a dose of 0.6 mg compared to 0.8 mg CQ (Table I). In addition, it was found – again – that the combinations of anti-mRBC IgG and lipCQ or CQ were more effective than anti-mRBC Fab'-lipCQ at equal CQ and protein concentrations.

TABLE IV

Effect of Fab'-lipCQ, IgG/lipCQ or IgG/CQ administration on the development of a patency after intravenous infection with synchronized parasitized mRETS

Treatment	Mean day of patency ± S.D. of positive rats	Number of rats radically cured [a]
anti-mRBC Fab'-lipCQ [b]	9.5 ± 0.6	2 (6)
anti-mRBC IgG/lipCQ	– [c]	4 (4)
anti-mRBC IgG/CQ	–	4 (4)
CQ	5.3 ± 0.5	0 (4)

[a] No parasites were detected during a 28 days period in rats infected with 10^5 p-mRETS (i.v.).

[b] CQ was given in a dose of 0.6 mg CQ per rat. IgG dose was 140 μg per rat in the indicated treatments.

[c] In the treated group no positive animal was obtained because all the treated animals in that group were radically cured.

Discussion

This study describes the application of a previously introduced in vivo model, to monitor specific targeting of immunoliposomes and antibodies (fragments) to their corresponding target cells [9]. The model is applied in the evaluation of the therapeutic efficacy of different antimalarial treatments.

Suspensions of Fab'-lipCQ could be prepared reproducibly and were stable during storage at least 6 months in terms of CQ leakage and particle size (particle aggregation or fusion was negligible).

In a preliminary experiment attempts were made to determine the effect of Fab' density of the liposomes on the elimination of mRBC from the blood compartment of the rat (Figs. 1 and 2). From these results it can be derived that there is a relation between Fab' density of the liposomes and elimination efficiency of the Cr-mRBC from the blood compartment of the rat. From the data in this report and the observation that ratios of 30.6 and 11.6 μg Fab'/μmol phospholipid exhibited similar clearance curves and organ distribution patterns [9] it was expected that Fab'-lip preparations with ratios over 12 μg Fab'/μmol phospholipid will not result in an improvement of the elimination of the Cr-mRBC. Nevertheless, in all therapeutic experiments anti-mRBC Fab'-lipCQ had a Fab' density of 25 ± 5 μg Fab'/μmol phospholipid.

This study further focused on the therapeutic application of CQ containing immunoliposomes in vivo. It is obvious that anti-mRBC antibodies (Fab-lip or IgG) alone are not able to suppress the infection significantly, despite the fact that they effectively remove the host cell of the parasite from the circulation (Figs. 1 and 2 and Ref. 9). The opsonization of the host cell by antibody and subsequent deposition in liver and spleen, presumably in the mononuclear phagocyte system (MPS) of these organs, did not lead to a sufficiently high level in inactivation of the parasites. They apparently continued to mature, form schizonts and merozoites, and reinvade new host cells causing infections as observed in buffer-treated animals. Assuming a parasite proliferation rate of 10 per cycle (24 h), a delay in patency of one day implies a 10-fold reduction in the number of parasites in the infection inoculum. Thus, more than 90% of the injected p-mRBC must be removed and inactivated by antibody in order to give a minimal delay (one day) in patency. In addition, the elimination from the blood stream takes some time (Fig. 1). Formation of

schizonts and merozoites, that will start a new infection in rat host cells, with no 'affinity' for the antibody, might occur during this period. This might explain the better results obtained with syn-p-mRETS (see below).

On the other hand, antibody treatment significantly potentiated the therapeutic effect of CQ or lipCQ. An accumulation of CQ from lipCQ in the MPS was to be expected because of the preferential deposition of liposomes in the MPS organs. This might enhance the interaction of CQ with opsonized p-mRBC that are also localized in these organs (Fig. 2). The question is whether the same holds true for free CQ. Further information on the deposition of free CQ in rats is needed. Previous experiments have shown that in the treatment of an ongoing infection in mice, lipCQ is superior to free CQ [16]. This was not found in the experiments described here: the way CQ was administered was not critical under the conditions chosen in the experiments presented in this study.

The role of specific anti-mRBC IgG in the potentiation of CQ is underlined by the observation that a lower amount of antibody at equal CQ concentration reduced the efficacy of the combination.

Agrawal et al. [20] showed a significant decrease of parasitemia 24 h after i.v. injection of CQ containing anti-mRBC $F(ab')_2$ liposomes in *P. berghei* infected mice. The effect was only temporarily; at later time points parasitemia levels of the experimental group caught up with those of the control groups receiving free CQ or lipCQ. These results can be explained by the fact that in their system (in contrast to our animal model) the anti-mRBC $F(ab')_2$ fragments had a low specificity; they interacted not only with the p-mRBC, but with all mRBC. Besides, Agrawal et al. [20] studied effects in animals with 1 to 2% infected mRBC (i.e., approx. 10^8 p-mRBC per ml blood). In the present study much lower infection levels were studied. As CQ acts at the parasitic level, antiparasitic effects are therefore dose dependent.

A common problem in our study and the study of Agrawal et al. [20] is the fact that both types of immunoliposomes do not bind to free parasites (merozoites). The antibodies used in these targeting experiments are directed against the surface of the host cell of the parasite, the mRBC. When parasites escape before targeting is completed they will start an infection in rat RBC, which can not be opsonized by the antibody. In that case suppression in the infection depends on the availability of CQ. It was therefore of interest to test the system with syn-p-mRETS. Particularly the absence of schizonts that rupture and release free living merozoites, which are not attacked by this antibody is important. Indeed, the results using syn-p-mRETS (predominantly trophozoite stages of the parasite) showed an increased therapeutic efficiency of anti-mRBC Fab'-lipCQ (Tables I and III). A proportion of the rats was radically cured with the same dose of CQ that only temporarily suppressed parasitemia when non-synchronous mRBC were used. These results support the hypothesis that in the case of non-synchronous mRBC the chance of escape of parasites is higher than with syn-p-mRETS. This in turn suggests that antibodies directed against the parasite and against all stages of the p-mRBC should be included in a formulation when antibody dependent targeting will be applied.

In a direct comparison the use of anti-mRBC IgG and lipCQ or CQ instead of anti-mRBC Fab'-lipCQ was therapeutically more effective. However, the hemagglutination titer of anti-mRBC Fab'-lip(CQ) was higher than that of anti-mRBC IgG at equal protein concentration (results not shown). At the moment it can only be speculated why anti-mRBC IgG/lipCQ and IgG/CQ combinations are therapeutically more effective than anti-mRBC Fab'-lipCQ. A reason may be that Fab'-lipCQ are eliminated too rapidly from the blood stream; they may be removed before they can interact with all target cells.

In summary, this report describes a suitable in vivo model for the analysis of antibody-mediated drug targeting with (immuno)liposomes. The synergism between specific antibody and CQ in the suppression of an infection is remarkable and may be of therapeutic significance.

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References

1 Tomlinson, E. (1987) Adv. Drug Deliv. Rev. 1, 87–198.
2 Toonen, P.A.H.M. and Crommelin, D.J.A. (1983) Pharm. Weekbl. Sci. Ed. 5, 269–280.
3 Torchilin, V.P. (1985) CRC Crit. Rev. in Therapeutic Drug Carrier Systems 2, 65–115.
4 Connor, J., Sullivan, S. and Huang, L. (1985) Pharmacol. Ther. 28, 341–365.
5 Sullivan, S.M., Connor, J. and Huang, L. (1986) Med. Res. Rev. 6, 171–195.
6 Leserman, L. and Machy, P. (1987) in Liposomes from Biophysics to Therapeutics (Ostro, M.J., ed.), pp. 157–194 Marcel Dekker, Inc., New York.
7 Crommelin, D.J.A. and Storm, G. (1989) in Comprehensive Medicinal Chemistry (Taylor, J.D., ed.), Vol. 5, Pergamon Books, Oxford, in press.
8 Peeters, P.A.M., Storm, G. and Crommelin, D.J.A. (1987) Adv. Drug Deliv. Rev. 1, 249–266.
9 Peeters, P.A.M., Oussoren, C., Eling, W.M.C. and Crommelin, D.J.A. (1988) Biochim. Biophys. Acta 943, 137–147.
10 Eling, W.M.C. (1977) Trans. Roy. Soc. Trop. Med. Hyg. 71, 267–268.
11 Mons, B., Janse, C.J., Croon, J.J.A.B. and Van der Kaay, H.J. (1983) Int. J. Parasitol. 13, 213–217.

12 Janse, C.J., Mons, B., Croon, J.J.A.B. and Van der Kaay, H.J. (1984) Int. J. Parasitol. 14, 317–320.

13 Martin, F.J. and Papahadjopoulos, D. (1982) J. Biol. Chem. 257, 286–288.

14 Szoka, F. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA 75, 4194–4198.

15 Peeters, P.A.M., Claessens, C.A.M., Eling, W.M.C. and Crommelin, D.J.A. (1988) Biochem. Pharmacol. 37, 2215–2222.

16 Peeters, P.A.M., Huiskamp, C.W.E.M., Eling, W.M.C. and Crommelin, D.J.A. (1989) Parasitology, in press.

17 Fiske, C. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–400.

18 Peterson, G.L. (1977) Anal. Biochem. 83, 346–356.

19 Heath, T.D., Macher, B.A. and Papahadjopoulos, D. (1981) Biochim. Biophys. Acta 640, 66–81.

20 Agrawal, A.K., Singhal, A. and Gupta, C.M. (1987) Biochem. Biophys. Res. Commun. 148, 357–361.