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Immunospecific targeting of immunoliposomes, F(ab′)$_2$ and IgG to red blood cells in vivo

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In this report a model to study the fate of target cells in the blood circulation after injection of appropriate immunoliposomes is discussed. The effect of intravenous administration of antimouse RBC immunoliposomes, F(ab′)$_2$ or IgG on the fate of intravenously injected ^{51}Cr-labelled mouse RBC (Cr-mRBC) in the mouse and, particularly, in the rat was studied. The immunoliposome was of the Fab′-MPBPE-REV type (Fab′-fragments covalently linked to reverse phase evaporation vesicles by maleimido-4-(*p*-phenylbutyrate)phosphatidylethanolamine). In the rat model a high blood level (80%) of the injected dose of target cells, Cr-mRBC, was maintained for several hours. The elimination by Fab′-liposomes, F(ab′)$_2$ or IgG of Cr-mRBC, and subsequent uptake into liver and spleen was dose dependent. Administration of Fab′-liposomes or F(ab′)$_2$ resulted in a preferential uptake into the spleen (above a certain dose also, but much lower, uptake into the liver was observed), while after IgG administration ^{51}Cr-label was mainly recovered in the liver. At equal protein doses (±130 μg) Fab′-liposomes induced a faster elimination of the Cr-mRBC and a higher uptake into the spleen than F(ab′)$_2$. The potential advantage of the use of drug-loaded immunoliposomes to eliminate target cells from the blood stream and to induce a certain pharmacological effect in the target cells, in comparison with the free antibody administration of F(ab′)$_2$ or IgG is discussed.

Introduction

The last decade immunoliposomes (liposomes coated with antibodies) have been studied extensively as potential drug or enzyme carriers for site-specific delivery in the biophase [1–3]. In vitro studies have been performed on the interaction of these immunoliposomes with their target cells [4–13]. Only a few reports discussed the possibility to direct these immunoliposomes to specific cells, particularly cancer cells, or tissues in vivo [14,15]. Because immunoliposomes larger than 0.2 μm can not pass cell barriers, active targeting with immunoliposomes in vivo can normally only be successful to cells or molecules located in the blood stream, the lymphatic system or in some cavities such as the peritoneal cavity, the bladder or uterus [1,9,16–18].

Recently some reports appeared on targeting of immunoliposomes [17] or antibody-coated microparticles [16] to specific cells in the bloodstream. Target cells were injected prior to the administra-

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tion of the antibody coated particulate system. As pointed out by the authors [16,17] the half-life of the i.v. injected target cells was too short (even without injecting the specific immunoliposomes) and consequently the background values of the radioactivity in the organs of the mononuclear phagocyte system (due to the initial spontaneous elimination of the target cells) were so high that there was no significant difference when immunoliposomes were injected. Thus no definite conclusions on the possible consequences and/or mechanisms of targeting of immunoliposomes [17] or microparticles [16] to specific target cells in the blood stream could be drawn.

This study describes a simple in vivo model to study the immunospecific elimination of ^{51}Cr-labelled mouse red blood cells (Cr-mRBC) from the blood stream and uptake in spleen and liver after i.v. injection into rats. The effect of subsequent i.v. administration of anti-mRBC-immunoliposomes on the disposition of the Cr-mRBC was studied and these results were compared with the results obtained after i.v. administration of free antibody (IgG) or antibody fragments ($F(ab')_2$). To our knowledge this is the first in vivo model where high levels of i.v. injected target cells (> 80% of the injected dose) were maintained in the blood stream during at least 4 h. As immunoliposomes, Fab'-MPBPE-REV [5] were used throughout this study.

Materials and Methods

Chemicals. Phosphatidylcholine (PC), phosphatidylserine (PS), cholesterol, pepsin and dithiothreitol (DTT) were purchased from Sigma Chemicals (St Louis, MO, U.S.A.). Phosphatidylethanolamine was obtained from Lipid Products (Nutfield, U.K.).

Succinimidylmaleimido-4-(*p*-phenylbutyrate) (SMPB) was obtained from Pierce Chemical Company (Rockford, U.S.A.). Sephadex gels and Protein A-Sepharose CL-4B were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Silicagel (70–325 mesh) was obtained from Merck (Darmstadt, F.R.G.) and SE-23 from Servacell (Heidelberg, F.R.G.). Carboxyfluorescein was purchased from Eastman Kodak Company (Rochester, N.Y., U.S.A.) and was purified by the method described by Ralston et al. [19]. $Na_2{}^{51}CrO_4$ was obtained from Amersham, U.K.

Animals and mRBC. Closed colony bred male Swiss mice (15–18 g) and male Wistar rats (140–170 g) were obtained from colonies of the animal facility of the University of Nijmegen. mRBC (from male Swiss mice) were purified by applying the heparinized whole blood onto a column containing three volumes of Sephadex G-150 Superfine and one volume of SE-23 to remove white blood cells [20].

Phagocytosis assay. Peritoneal exudate cells from normal male Swiss mice were harvested by peritoneal lavage as described by Conrad [21]. Peritoneal cells were washed once in RPMI 1640 containing heparin (5 units/ml) and 10% fetal calf serum (FCS). Samples of 1 ml (about $5 \cdot 10^5$ cells/ml) were pipetted into tissue culture clusters (24 wells, Costar) containing 15 mm round coverslips (Thermanox 5414, Miles Laboratories) with a bent edge to facilitate manipulation. The cells were allowed to adhere to the coverslips for at least 2 h at 37°C. After incubation non adherent cells were washed by gently moving the coverslips in warm saline solution (37°C). Quantitative analysis revealed that approx. $2 \cdot 10^5$ macrophages adhered onto each coverslip. Washed coverslips were placed into wells containing 1 ml mRBC (10^7/ml per well) which were either preincubated with liposomes or Fab'-liposomes. Suspensions were incubated for 90 min at 37°C. Non-phagocytozed cells were removed by gently washing the coverslips in warm saline solution [22].

Preparation of maleimido-4-(p-phenylbutyrate) phosphatidylethanolamine bearing reverse phase evaporation vesicles. Maleimide-4-(*p*-phenylbutyrate)phosphatidylethanolamine (MPBPE) was prepared as described earlier [5]. MPBPE bearing reverse phase evaporation vesicles were prepared by the method of Szoka and Papahadjopoulos [23] with minor modifications [13] and a lipid composition of: cholesterol/PC/PS/MPBPE 10:10:1:0.5. The vesicle dispersion (in a buffer containing 100 mM sodium acetate and 88 mM sodium chloride, pH 7.4) was extruded through 0.4 μm and subsequently through 0.2 μm polycarbonate membranes (Nucleopore Corp, Pleasanton, U.S.A.). In cases where carboxyfluorescein was

entrapped in the MPBPE bearing reverse phase evaporation vesicles, 100 mM carboxyfluorescein in 10 mM Tris buffer (pH 7.4) was used instead of buffer alone. After extrusion, free carboxyfluorescein was separated from liposomal carboxyfluorescein by gel filtration chromatography on a Sephadex G-50 fine column (0.8 × 20 cm), pre-equilibrated with buffer.

Preparation of antibody and its fragments. Anti-mRBC antibodies were raised in New Zealand white albino rabbits. They received i.v. injections of 10^9 mRBC repeatedly until high haemagglutination (HA) titers in serum were obtained: 2^{16} in a serial twofold dilution test [24]. IgG was isolated by Protein A-Sepharose CL-4B affinity chromatography [25]. Rabbit anti-mRBC $F(ab')_2$ and Fab′ were prepared and purified as described earlier [4], except that nitrogen instead of argon was used and the buffer used for PD-10 gelchromatography, to remove DTT from the Fab′-fragments, had a pH 6.5. The HA titers of the isolated IgG and $F(ab')_2$ were 2^{15} and 2^{12}, respectively, at a protein concentration of 9 mg/ml.

Coupling of Fab′ fragments to the MPBPE bearing reverse phase evaporation vesicles. Freshly prepared MPBPE bearing reverse phase evaporation vesicles (final concentration 3.5 μmole phospholipid/ml in deoxygenated buffer were mixed wit freshly prepared Fab′ fragments (final concentration 0.3 mg/ml). The coupling reaction was carried out in a nitrogen atmosphere under stirring at room temperature for 90 min. Fab′-MPBPE bearing reverse phase evaporation vesicles (Fab′-liposomes) were separated from unconjugated Fab′-fragments by ultracentrifugal sedimentation of the Fab′-liposomes at 80000 × *g* during 45 min in a Beckman TY-65 rotor [13].

In vitro cell binding assay for Fab′-liposomes. A 2.5% suspension of purified, washed mRBC was mixed with an equal volume of carboxyfluorescein containing Fab′-liposomes, which had been preincubated with 6.5 mM DTT (to avoid non-specific interactions of unoccupied MPBPE molecules on the liposome) for 30 min at room temperature, and the amount of specifically bound Fab′-liposomes was determined as described earlier [13]. Briefly, the mixture was incubated at 37 °C for 30 min. The mRBC were washed twice with ice-cold buffer and after centrifugation (600 × *g*, 5 min), to separate mRBC bound Fab′-liposomes from unbound Fab′-liposomes. the resulting pellet was resuspended in 1 ml buffer containing 0.5% Triton X-100 (v/v) to lyse the Fab′-liposomes bound to the mRBC, thereby releasing the entrapped carboxyfluorescein. The suspension was heated for 20 min at 80 °C to denature hemoglobin and other proteins. After centrifugation at 1500 × *g* (10 min) the supernatant was analysed for carboxyfluorescein.

In vivo experiments. mRBC (10^9/ml buffer) were labelled with $Na_2{}^{51}CrO4$ (about 10 μCi/5 · 10^8 mRBC) during 60 min at 37 °C, washed twice, resuspended in buffer and used immediately. In the experiments performed in mice the Cr-mRBC were incubated with Fab′-liposomes as described in the previous section and the ^{51}Cr-labelled mRBC with adhering Fab′-liposomes (F-lip-Cr-mRBC) ($1.5 \cdot 10^8$ per mouse) were injected i.v. into the tail vein of mice. In additional experiments either 5 μmole Fab′-liposome (21.5 μg Fab′/μmole phospholipid) or buffer were injected i.v. 45 min after the injection of Cr-mRBC. Doses of about 300000 cpm were given per mouse; the total volume injected into the mice was always 0.2 ml. In the rat experiments Cr-mRBC ($1.5 \cdot 10^9$ equivalent to about 450000 cpm per rat) were injected i.v. in the tail vein of the rat, followed by the indicated amount of Fab′-liposomes, liposomes, IgG, $F(ab')_2$ or 0.5 ml buffer 30 to 45 min later. At time intervals, blood samples were taken (20 or 50 μl for mouse or rat, respectively) from the tail vain into heparinized glass capillaries and the blood was transferred to scintillation vials and counted for ^{51}Cr radioactivity. Total injected radioactivity (= 100%) was determined by counting a sample of the injected material and the proportion of radioactivity in the circulation at the indicated time points was calculated assuming a total blood volume of 6% with respect to the body weight [26]. At the indicated time points the animals were killed and radioactivity in liver, spleen and lungs was determined. The sum of radioactivity in blood, spleen, liver and lungs refers to total recovery in the results section. Each data point is the mean ± S.D. from three animals, unless otherwise stated.

Other methods used. Protein was determined by

the method of Peterson [27]. Lipid phosphate was estimated by the colorimetric method of Fiske and SubbaRow [28]. The quality of the phospholipids and synthesized MPBPE was tested by a TLC method [29].

Carboxyfluorescein fluorescence was measured at 516 nm in a Kontron Instruments spectrofluorimeter, model SFM 25 (Zurich, Switzerland) at an excitation wavelength of 489 nm.

Particle size analysis was performed by dynamic light scattering with a Malvern PCS 100 SM (Malvern Ltd. Worcestershire, U.K.) equipped with a particle analyzer processor (model 7027) and a 27 mWatt helium/neon laser (NEC Corp, Tokyo, Japan).

Results

The interaction of mRBC preincubated with liposomes or Fab′-liposomes with macrophages

In a pilot experiment the binding of Fab′-liposomes to mRBC was measured. Fab′-liposomes (0.06 μmole phospholipid, ratio 22.6 μg Fab′/μmole phospholipid and diameter 0.32 μm) containing 100 mM carboxyfluorescein were incubated with 10^7 mRBC for 30 min at 37°C. Under these circumstances 7% of the originally added amount of Fab′-liposomes was bound to the mRBC (Fig. 1); this is equivalent to about 600 Fab′-liposomes per mRBC (the procedure for calculation was described previously: Peeters et al. [13]). Under comparable conditions only 0.2% of the liposomes bound to mRBC. In a six hour experiment the binding between Fab′-liposomes and mRBC appeared to be stable (results not shown). Subsequently it was established whether mRBC with bound Fab′-liposomes were recognized by macrophages. Adherent mouse peritoneal cells ($2 \cdot 10^5$) on coverslips were incubated with mRBC (10^7) which had been preincubated either with liposomes (0.06 μmole phospholipid and diameter 0.29 μm) or Fab′-liposomes (0.06 μmole phospholipid, 22.6 μg Fab′/μmole phospholipid; diameter of 0.32 μm) at 37°C for 30 min. The results showed that mRBC preincubated with liposomes were not bound to the macrophages (Fig. 2A) but mRBC with adherent Fab′-liposomes were avidly bound to the macrophages (Fig. 2B).

Distribution of ^{51}Cr-labelled mRBC with adhering Fab′-liposomes, and targeting of Fab′-liposomes to mRBC in mice

The above described in vitro results show a high level of interaction between immunoliposome-opsonized mRBC and mouse macrophages. Next the distribution of F-lip-mRBC in vivo was studied in mice. Cr-mRBC were incubated with Fab′-liposomes as described in the previous section, and the complex was injected intravenously. Cr-mRBC preincubated with liposomes (without Fab′-fragments) served as controls. Blood clearance was monitored directly after injection (Fig. 3) and the organ distribution was determined 3 h after injection (Table I). Clearance from the bloodstream is rapid and radioactivity localized predominantly in liver and spleen.

When the Cr-mRBC were injected first and the Fab′-liposomes 30 min later the elimination process was less efficient (Fig. 3) and so was the uptake in liver and spleen (Table I). Obviously the Fab′-liposomes injected after the Cr-mRBC are targeted to all mRBC and the number of Fab′-liposomes per mRBC in vivo is therefore substantially reduced, compared to the experiment where the mRBC were preincubated with Fab′-liposomes in vitro.

On the one hand the clearance and organ uptake of F-lip-Cr-mRBC could be in line with the in vitro observations: the opsonized mRBC are recognized and taken up by the mononuclear phagocyte system of the liver and spleen. On the other hand the aim is to target to a specific population of cells in the bloodstream and for that reason the model was adapted.

Distribution of ^{51}Cr-labelled mRBC in rats after targeting of Fab′-liposomes to mRBC

A common problem in studying the interaction of immunoliposomes with their target cells in the blood compartment is that usually these, sometimes modified, target cells are very rapidly eliminated from the blood after i.v. administration [16,17]. Therefore, there is a need for an in vivo model in which a large fraction of the target cells (80–90% of the injected dose) will circulate over a period of several hours.

To restrict the binding of Fab′-liposomes to previously injected Cr-mRBC, the experiments

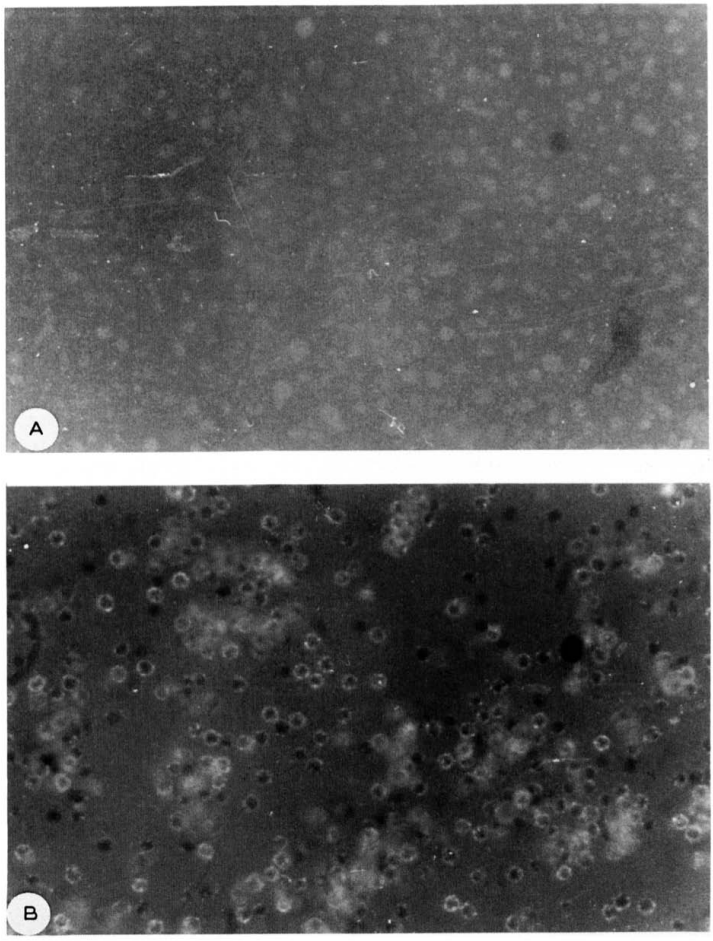

Fig. 1. Fluorescent microphotographs (magnification: 2070×) of the binding of liposomes (A) and Fab′-liposomes (B) to mRBC. Whole heparinized mouse blood (0.25 ml equivalent to $2 \cdot 10^9$ mRBC) was incubated with 0.25 ml liposome (4 μmol phospholipid and diameter 0.29 μm) or Fab′-liposome suspension (4 μmole phospholipid, 22.6 μg Fab′/μmole phospholipid and diameter 0.32 μm), which were preincubated with DTT for 30 min at 37°C. mRBC were isolated and smears were examined by a fluorescence microscope. Liposomes and Fab′-liposomes used in this experiment contained 10 mM carboxyfluorescein.

were performed in rats. Clearance of Cr-mRBC in rats is slow (Fig. 4). After 3 h 80% of the injected amount of Cr-mRBC is still circulating. Clearance can be enhanced considerably when Fab′-liposomes are injected. Increasing the dose of Fab′-liposome from 5.3 to 21.3 μmole phospholipid enhanced the blood clearance of mRBC in a dose dependent way (Fig. 4). Again liver and spleen are

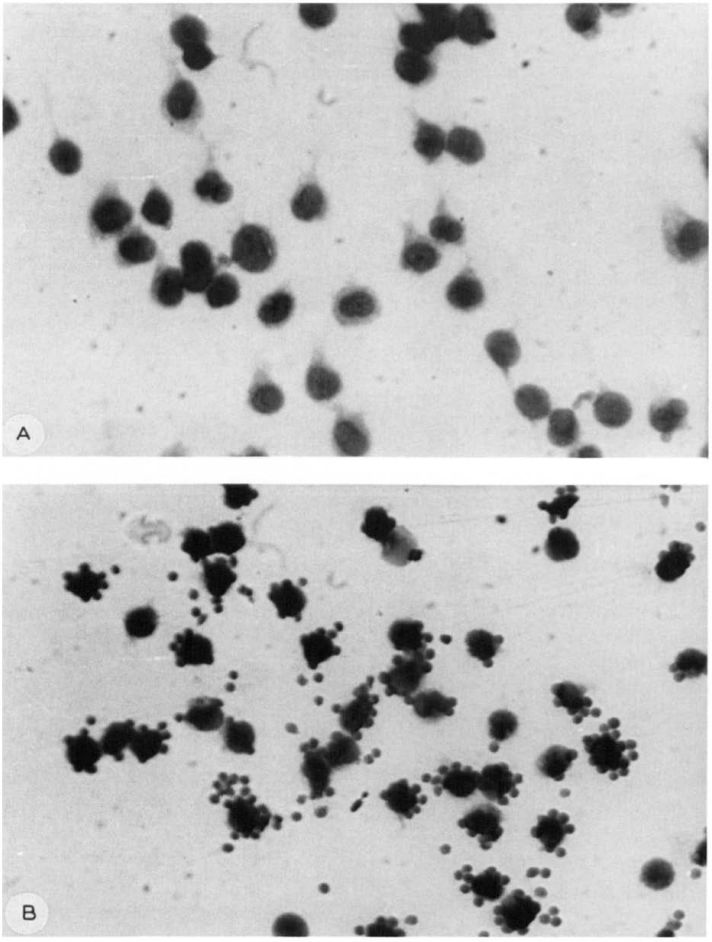

Fig. 2. Phase photomicrographs (magnification: 1530×) of the interaction between mouse peritoneal macrophages ($2 \cdot 10^5$) and mRBC which were either preincubated with liposomes (A) or Fab′-liposomes (B). Liposomes (0.06 μmole phospholipid) and Fab′-liposomes (0.06 μmole phospholipid) were preincubated with 6.5 mM DTT during 30 min at room temperature before they were incubated with mRBC (10^7) at 37 °C during 30 min. Non-interacting liposomes and Fab′-liposomes were removed by centrifugation (600 × *g*, 5 min) and the resulting mRBC suspension (10^7) were incubated with adherent mouse peritoneal macrophages for 90 min at 37 °C in a total volume of 1 ml. Non-bound cells were removed by gently washing the coverslips in warm saline solution. Specifications of liposomes and Fab′-liposomes are the same as described in the legends of Fig. 1.

important organs for the uptake of Cr-mRBC-Fab′-liposome complexes (Table II). A reduction of total recovery is observed when the highest dose (21.3 μmole Fab′-liposomes) is injected (Table II). Liposomes alone do not affect the clearance of mRBC (Fig. 4).

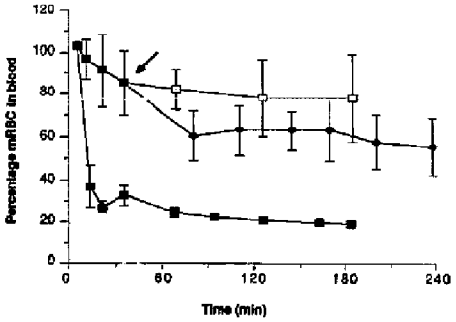

Fig. 3. Blood level of Cr-mRBC ($1.5 \cdot 10^8$) in mice after i.v. administration of buffer (□) or 5 μmole Fab′-liposomes (◆) 30 min after injection of Cr-mRBC (arrow). ^{51}Cr-level at $T = 0$ is taken as 100%; (■) refers to mice injected with F-lip-Cr-mRBC ($1.5 \cdot 10^8$ and about 600 Fab′-liposomes per RBC) at $T = 0$. The Fab′-liposomes used had a ratio of 21.5 μg Fab′/μmole phospholipid and a diameter of 0.38 μm. The total volume injected was 0.2 ml in all cases. Each data point represents the mean ± S.D. of three mice. Small S.D. values are not shown.

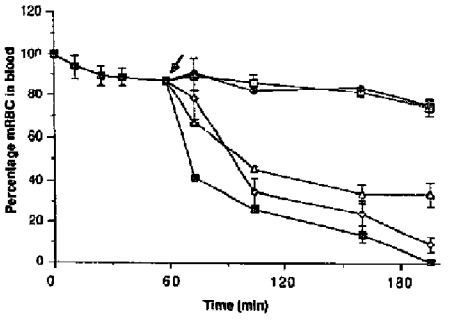

Fig. 4. Blood level of Cr-mRBC ($1.5 \cdot 10^9$) in rats after i.v. administration of buffer (□), liposomes (21.1 μmole phospholipid per rat, (◆), or Fab′-liposomes at three doses: 5.3 (△), 10.7 (◇) and 21.3 (■) μmole phospholipid per rat, 50 min after injection of Cr-mRBC (arrow). Fab′-liposomes had a ratio of 30.6 μg Fab′/μmole phospholipid and a diameter of 0.38 μm, while liposomes had a diameter of 0.28 μm. In all cases a total volume of 0.5 ml was injected. Each data point represents the mean ± S.D. of three rats. Small S.D. values are not shown.

In an additional experiment Fab′-liposomes were used, however, with a 3-fold lower concentration of Fab′ per μmole phospholipid. Again a dose dependent clearance and organ uptake of Cr-mRBC was found (results not shown). In addition, the effect on clearance and organ uptake of comparable doses of Fab′-liposomes was the same.

Distribution of ^{51}Cr-labelled mRBC in rats after targeting of F(ab′)$_2$ or IgG to mRBC

The same experimental model (circulating Cr-mRBC in rats) was used to study the effect of F(ab′)$_2$ (the clearance and organ uptake is Fc-independent as with Fab′-liposomes) or IgG. The dose of F(ab′)$_2$ varied from 60 to 360 μg. The clearance of mRBC was enhanced by increasing doses of F(ab′)$_2$ (Fig. 5). The clearance rate is comparable to that of Fab′-liposomes (Fig. 4). At 120 and 180 μg doses of F(ab′)$_2$ there was a preferential uptake of label by the spleen (Table III). With higher doses liver uptake caught up with spleen uptake; after injection of 360 μg F(ab′)$_2$ spleen uptake decreased to control levels, liver uptake was comparable to the situation after injection of 240 μg F(ab′)$_2$ and a marked reduction of the total recovery of label was observed.

In the same model, the effect of IgG (doses ranging from 10 μg to 360 μg IgG) administration

TABLE I

DISTRIBUTION OF Cr-mRBC IN MICE OVER VARIOUS ORGANS, BLOOD AND TOTAL RECOVERY OF ^{51}Cr-LABEL AFTER VARIOUS TREATMENTS

Distribution of Cr-mRBC in mice over spleen, liver, lungs, blood and total recovery of ^{51}Cr-label 3 h after i.v. administration of Cr-mRBC, followed by buffer (Control group) or 5 μmole Fab′-liposomes (in vivo group) at the indicated time in Fig. 3 (arrow), or F-Lip-Cr-mRBC (in vitro group) at $T = 0$. For specifications of Fab′-liposomes and further experimental details see legends of Fig. 3.

Group	Blood	Spleen	Liver	Lungs	Total
Control	75.8 ± 21.0	1.3 ± 0.2	3.1 ± 0.4	1.7 ± 0.6	81.9 ± 22.0
In vivo	53.1 ± 13.7	4.5 ± 1.7	3.1 ± 0.5	1.7 ± 0.4	62.4 ± 15.9
In vitro	16.2 ± 1.1	17.5 ± 2.8	39.1 ± 7.8	0.6 ± 0.1	73.4 ± 5.0

TABLE II

DISTRIBUTION OF Cr-mRBC IN RATS OVER VARIOUS ORGANS, BLOOD AND TOTAL RECOVERY OF ^{51}Cr-LABEL AFTER i.v. ADMINISTRATION OF VARIOUS DOSES OF Fab'-LIPOSOMES AND CONTROLS

Distribution of Cr-mRBC in rats over spleen, liver, lungs, blood and total recovery of ^{51}Cr-label 2.5 h after i.v. administration of buffer, liposomes (21.1 mole phospholipid per rat) and Fab'-liposomes at three dosage levels: 5.3, 10.7 and 21.3 μmole phospholipid per rat. For further details on liposomes or Fab'-liposomes: see legends of Fig. 4.

Treatment	Blood	Spleen	Liver	Lungs	Total
Buffer	74.9±3.9	8.2±0.2	11.5±0.3	4.4±0.6	99.0± 2.0
Liposomes	76.0±2.0	4.1±1.1	9.9±1.0	2.0±0.2	91.9± 2.3
5.3 μmole Fab'-lip	33.5±5.5	39.1±2.0	16.4±5.4	2.1±0.2	90.9± 1.7
10.7 μmole Fab'-lip	9.0±4.0	53.4±8.4	29.5±9.6	1.4±0.3	93.4±13.8
21.3 μmole Fab'-lip	1.0±0.2	53.0±7.0	31.5±9.8	1.2±0.2	86.1± 2.0

TABLE III

DISTRIBUTION OF Cr-mRBC IN RATS OVER VARIOUS ORGANS, BLOOD AND TOTAL RECOVERY OF ^{51}Cr-LABEL AFTER i.v. ADMINISTRATION OF $F(ab')_2$

Distribution of Cr-mRBC in rats over spleen, liver, lungs, blood and total recovery of ^{51}Cr-label 1.5 h after i.v. administration of the indicated $F(ab')_2$ doses per rat in a total volume of 0.5 ml. Each data point represents the mean±S.D. of three rats.

$F(ab')_2$ (μg)	Blood	Spleen	Liver	Lungs	Total
60	86.2±4.3	12.7±9.0	12.4± 5.3	5.3±0.6	103 ± 5.3
120	35.7±7.1	36.4±4.8	14.0± 2.3	2.8±0.5	86.3± 0.8
180	25.7±4.1	45.5±3.2	10.1± 0.5	3.1±0.2	84.4± 0.6
240	12.8±8.7	37.5±9.1	30.2±12.5	2.2±2.2	82.7± 6.2
360	6.8±0.5	4.8±2.1	35.8±12.5	0.7±0.7	48.0±14.3

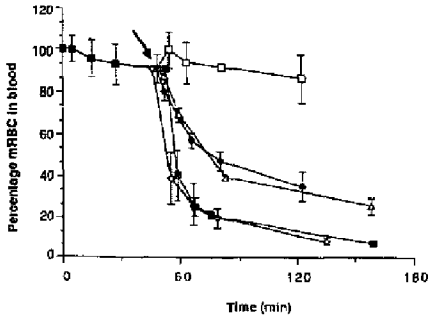

Fig. 5. Blood level of Cr-mRBC ($1.5 \cdot 10^9$) in rats after i.v. administration of different $F(ab')_2$ doses, 50 min after injection of Cr-mRBC (arrow) in a total volume of 0.5 ml: 60 (□), 120 (◆), 180 (△), 240 (◇) and 360 (■) μg $F(ab')_2$ per rat. Each data point represents the mean±S.D. of three rats. Small S.D. values are not shown.

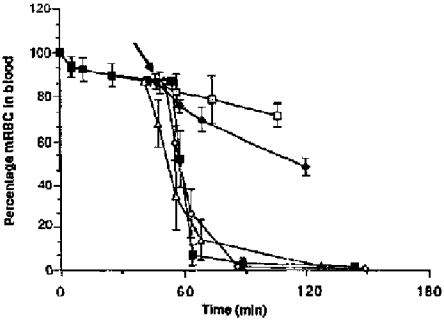

Fig. 6. Blood level of Cr-mRBC ($1.5 \cdot 10^9$) in rats after i.v. administration of different IgG doses, approx. 50 min after injection of Cr-MRBC (arrow) in a total volume of 0.5 ml: 10 (□), 30 (◆), 90 (△) $n = 5$ 180 (◇) and 360 (■) $n = 4$ μg IgG per rat. Each data point represents the mean±S.D. of three rats (unless otherwise indicated). Small S.D. values are not shown.

TABLE IV

DISTRIBUTION OF Cr-mRBC IN RATS OVER VARIOUS ORGANS, BLOOD AND TOTAL RECOVERY OF ^{51}Cr-LABEL AFTER i.v. ADMINISTRATION OF IgG

Distribution of Cr-mRBC over spleen, liver, lungs, blood and total recovery of ^{51}Cr-label 1.5 h after i.v. administration of the indicated IgG doses per rat in a total volume of 0.5 ml. Each data point represents the mean ± S.D. of three rats (unless otherwise indicated).

IgG (μg)	Blood	Spleen	Liver	Lungs	Total
10	69.9±8.5	6.3±1.4	7.6±0.7	1.2±0.1	84.9±10.3
30	48.2±4.0	27.4±6.2	8.2±0.4	0.8±0.6	84.8± 2.4
90 (n = 5)	1.2±1.4	19.6±6.8	59.5±9.0	0.8±0.7	81.6± 6.7
180	0.7±0.7	19.2±3.4	59.8±2.7	1.8±0.2	81.5± 6.3
360 (n = 4)	1.5±1.3	13.0±2.5	70.4±6.0	1.2±0.8	86.4± 6.3

on the Cr-mRBC elimination (Fig. 6) and subsequent uptake into liver and spleen (Table IV) was studied. Again elimination of Cr-mRBC from the bloodstream, and subsequent spleen and liver uptake were dose dependent. At relatively low doses (30 μg IgG per rat) mainly spleen uptake occurred (Table IV). However, at doses of 90 μg IgG or higher, Cr-mRBC were preferentially cleared by the liver while the relative spleen uptake tends to decrease (Table IV).

Discussion

The experiments describe an in vivo model for specific targeting of Fab′-liposomes to the corresponding target cells. Fab′-liposomes avidly bind to erythrocytes in vitro (Fig. 1) as well as in vivo (Figs. 3 and 4) and are well recognized by the cells of the mononuclear phagocyte system in vitro (Fig. 2) as well as in vivo (Table I and II). The aim of specific targeting is to eliminate diseased cells from the bloodstream (e.g. erythrocytes infected by the malaria parasite). In the section: 'Distribution of ^{51}Cr-labelled mRBC with adhering anti-mRBC Fab′-liposomes, and targeting of Fab′-liposomes to mRBC in mice' the efficiency is restricted (Fig. 3 and Table I), apparently due to the fact that now all mRBC are recognized as targets, resulting in a considerable reduction of the amount of Fab′-liposomes per mRBC. The observation that mRBC can survive for a considerable period of time in rats (Fig. 4), allowed kinetic studies of specific targeting of Fab′-liposomes to Cr-mRBC in rats. Fab′-liposomes injected into rats apparently quickly bind to their target cells, causing a rapid removal from the circulation (Fig. 4). Fab′-liposome induced elimination of target cells is dose dependent and can be more or less complete (Fig. 4). This model has a distinct advantage over the systems described so far. Until now, model systems suffered from a rapid spontaneous removal of the target cells from the bloodstream [37] which made it impossible to prove specific targeting and subsequent organ uptake [16]. The observed rapid clearance of mRBC after interacting with the appropriate immunoliposomes is in contrast to the data reported by Singhal and Gupta [30]. They did not observe enhanced clearance of ratRBC, to which anti-ratRBC $F(ab')_2$-liposomes were bound in vitro, after intravenous injection in rats. According to their data 6% of the added amount of $F(ab')_2$-liposomes was bound to ratRBC. Assuming that the diameter of their immunoliposomes was equal to that of their liposomes (45 nm) and assuming a surface area per phospholipid head (50% cholesterol included) of 0.96 nm^2 [23] about 5400 immunoliposomes per rat RBC should be bound during preincubation. In our experiments the binding of 600 immunoliposomes per mRBC was enough for rapid clearance in mice. The reason for this discrepancy is not clear. We have used immunoliposomes of the Fab′-MPBPE-REV type for several reasons [1,13]:

(a) Fab′-fragments on the liposomes are optimally orientated for antigen binding [5].

(b) The thioether bond between MPBPE bearing reverse phase evaporation vesicles and the

Fab′-fragment is stable under reducing circumstances, in serum [5] and in plasma (Ref. 13; Fig. 1, this study).

(c) The use of Fab′-fragments instead of whole IgG is supposed not to activate the Fc-receptor of macrophages and thus minimizing elimination of the Fab′-liposomes by the mononuclear phagocyte system [1,17].

The observations described in Fig. 4 and the additional experiment with the 3-fold lower concentration of Fab′ per μmole phospholipid (results not shown) show that, under the experimental conditions, the ratio of Fab′ molecules per liposome for opsonization and elimination of mRBC is not critical. At nearly equal protein concentrations Fab′-liposomes are more efficient in the clearance of mRBC than $F(ab')_2$ (Figs. 4 and 5). The data presented in Figs. 5 and 6 show that clearance of mRBC is more efficient in the case of IgG than for $F(ab')_2$. Opsonization with IgG provides the opportunity of Fc-binding by the cells of the mononuclear phagocyte system. The preferential uptake of IgG opsonized mRBC by the liver (Table IV) supports this [12]. The potential therapeutic advantages of the accumulation of opsonized target cells by the cells of the mononuclear phagocyte system will be discussed later.

Studies on the distribution of specifically opsonized mRBC in vivo showed that spleen and liver are the most important clearance organs, which is in line with their important role as scavenger organs of the elimination of circulating RBC on rheological grounds. The rheological changes after interaction with liposomes [31,32], as well as the subsequent enhanced uptake by the spleen [33] have been described before. Fab′-liposomes modify mRBC strongly because, despite the absence of the Fc-fragment they are avidly bound to macrophages (Fig. 2). At low doses of Fab′-liposomes, $F(ab')_2$, or IgG opsonized mRBC were predominantly taken up by the spleen. In particular $F(ab')_2$ efficiently directed mRBC to the spleen. This might be caused by a change in the rheological properties of the cells after binding of the specific antibody (fragment). At high doses, resulting in an increased number of $F(ab')_2$ per mRBC, the chances of an interaction with cells of th mononuclear phagocyte system greatly increase. In this respect a reduced blood flow in the liver sinusoids combined with a high number of phagocytic Kuppfer cells in the sinusoidal lining may explain the substantial uptake of mRBC in this organ under these conditions. Opsonization of the target cell or pathogen by immunoliposomes, even macrophage uptake of the immunoliposome-target cell or pathogen complex might not be sufficient to kill the target cell or pathogen. The encapsulation of a cytotoxic drug into the immunoliposomes is therefore desirable to achieve both successful targeting to and killing of the target; the encapsulated drug will be released from the immunoliposomes inside the macrophage after lysosomal degradation, enter the target cell and exert its therapeutic effect. An alternative approach with interesting therapeutic potential is the use of $F(ab')_2$ or IgG and liposomes separately: first eliminate target cells selectively from the bloodstream by $F(ab')_2$ or IgG administration and direct them to spleen and liver. Subsequently inject liposomes containing the therapeutic agent. These liposomes normally also end up in the macrophages in spleen and liver [14]. The phagocytosed target cells or pathogens will be attacked by the released drug from the liposomes taken up by the same macrophages. At the moment we are studying these two approaches for the inactivation of *Plasmodium berghei* infected mRBC in rats. The two approaches described above for drug targeting to RBC can have implications for the therapy of a number of diseases localized in the blood compartment. By injection of immunoliposomes or, alternatively, by sequential injection of IgG/$(Fab')_2$ and liposomes (indirect approach), the disposition – and fate – of thrombocytes, subset populations of T-lymphocytes or circulating tumor cells can be drastically changed.

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