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Antitumour activity of cytotoxic liposomes equipped with selectin ligand SiaLe^X, in a mouse mammary adenocarcinoma model

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Abstract

The overexpression of lectins by malignant cells compared with normal ones can be used for the targeting of drug-loaded liposomes to tumours with the help of specific carbohydrate ligands (vectors). Recently we have shown that liposomes bearing specific lipid-anchored glycoconjugates on a polymeric matrix bind *in vitro* to human malignant cells more effectively and, being loaded with a lipophilic prodrug of merphalan, reveal higher cytotoxic activity compared with unvectored liposomes. In this study, carbohydrate-equipped cytotoxic liposomes were tested *in vivo* in a mouse breast cancer model, BLRB-Rb (8.17)1Iem strain with a high incidence of spontaneous mammary adenocarcinoma (SMA). Firstly, a cell line of the SMA was established which was then used to determine the specificity of the tumour cell lectins. After screening of the lectin specificity of a number of fluorescent carbohydrate probes, SiaLe^X was shown to be the ligand with the most affinity, and a lipophilic vector bearing this saccharide was synthesised. Then different liposomal formulations of the synthetic merphalan lipid derivative and SiaLe^X vector were prepared and applied in the treatment of mice with grafted adenocarcinomas. The results of the tumorigenesis data show that the therapeutic efficacy of merphalan increases sharply after its insertion as a lipophilic prodrug into the membrane of SiaLe^X-vectored liposomes.

Keywords: Antitumour activity; Liposomes; Merphalan; Targeting; Glycoconjugates; Mouse mammary adenocarcinoma; SiaLe^X

1. Introduction

Liposomes as carriers for cytotoxic drugs continue to be extensively studied [1,2]. A noticeable reduction in the general toxicity of agents encapsulated in liposomes is brought about by changes in their distribution between tissues and organs. In addition, it has been shown that liposomes of medium size (100–120 nm in diameter) accumulate in malignant tumours owing to their high vascular permeability [3,4]. An increasing number of studies on liposomal drug delivery have been observed over the last 5 years. In particular, it has been shown that liposomes can be protected against opsonisation and from rapid clearance from the circulation by cells of the reticuloendothelial system. This can be done by grafting polyethyleneglycol (PEG) chains onto the liposomal surfaces (Stealth® liposomes) or by the inclusion of phosphatidylinositol or gangliosides, thus producing a highly hydrated protective shell on the membrane surface [5,6]. Interestingly, the superior therapeutic efficacy of doxorubicin included in the internal volume of PEG-protected liposomes has been shown in clinical trials [7,8]. In addition, sterical stabilisation of liposomes as drug carriers can be achieved by coating their surfaces with natural polysaccharides, after chemical modification of the latter with hydrophobic anchors (e.g. cholesterol) [9].

But a topical problem of the directed delivery of drug-loaded liposomes to malignant tumours remains, this has led to the equipping of liposomes with specific ligands (vectors). The most popular approach here is the use of immunoliposomes bearing tumour-specific antibodies [10–12]. Another approach that is less expensive avoids the immune reaction to the antibodies. This is

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based on the phenomenon of the overexpression of lectins (specific carbohydrate-binding proteins) by mammalian malignant cells compared with normal ones [13,14]. Recently we suggested targeting the cytotoxic liposomes to tumours by means of specific carbohydrate determinants (vectors), and showed that liposomes bearing lipid-anchored polymeric glycoconjugates bound *in vitro* to HL60 and human lung adenocarcinoma cells more effectively and, being loaded with a merphalan lipid derivative, revealed a higher cytotoxic activity was obtained compared with the unvectored liposomes [15]. Segawa and colleagues previously demonstrated that liposomes equipped with the lactose derivatised polysaccharide were taken up more abundantly by the liver cancer cells *in vitro* than lactose-free liposomes [16]. Such liposomes when loaded in the internal aqueous volume with doxorubicin restrained the growth of AH66 hepatoma in nude mice more effectively than liposomes without lactose [17].

Liposomes bearing a drug as a biodegradable lipid derivative (fatty acyl ester, diglyceride, etc.) in the bilayer, possess some attractive features: the drug leakage in the circulation presumably diminishes, as well as losses at the stage of liposome–cell interaction. Moreover, preparation of such liposomes is relatively simple. In cells, lipid derivatives being split by endogenous enzymes release initial antitumour agents (as a rule, hydrophilic compounds *per se*). A number of synthetic lipophilic prodrugs are known, e.g. derivatives of 5-fluorouridine [11], 1-β-arabinofuranosylcytosine (araC) [18] and doxorubicin [19]. In a previous study, we synthesised a series of lipid derivatives of sarcolysine (DL-melphalan, merphalan), rubomycin (daunorubicin) and methotrexate [20,21], and applied them in liposomal formulations to treat mice with experimental leukaemia P-388 [22] and mammary adenocarcinoma [23]. Positive results were obtained, specifically, a diglyceride derivative of merphalan applied as a liposomal formulation caused a 2-fold lifespan increase compared with treatment with the intact drug [22].

Herein we report the results of *in vivo* testing of carbohydrate-equipped liposomes loaded with the lipophilic prodrug of a well-known antitumour agent, merphalan, in a mouse spontaneous mammary adenocarcinoma model, which is often used as a model for human breast cancer.

2. Materials and methods

2.1. Reagents and chemicals

Phosphatidylcholine (PC) from egg yolk and phosphatidylinositol (PI) from *S. cerevisiae* were obtained from Reakhim (Moscow, Russia). 3-aminopropyl glycosides of Sialyl-Lewis^X [SiaLe^X, Neu5Acα2-3Galβ1-4(Fucα(1-3)-GlcNAcβ] and A-trisaccharide [A_{tri}, GalNAcα1-3(Fuc-α1-2)Galβ] were synthesised as previously described [24,25]. Fluorescent carbohydrate probes (34 specimens) in the form of glycosylated fluorescein-labelled polyacrylamide derivatives were a generous gift of Syntesome GmbH (Munich, Germany). SiaLe^X-vector was synthesised from 3-aminopropyl glycoside and Lubrol PX (Sigma, St Louis, MO, USA) as described for the other glycoconjugates [21]. The lipophilic derivative of DL-melphalan, octadecylmerphalan (C_{18}Mrph), was synthesised from 1-octadecanol and merphalan as the corresponding dioleoylglycerol derivative [20].

2.2. Tumour cells and assay of their carbohydrate specificity

A cell line was established by passaging of primary cultures produced from the tumours of BLRB-Rb (8.17)1Iem (hereafter called BLRB) mouse females. These have a more than 95% incidence of spontaneous mammary adenocarcinomas with subsequent, inevitable metastases in the lungs [23,26]. Tumour tissue was finely minced under sterile conditions, rinsed with Ca^{2+}/Mg^{2+}-free saline solution, and digested in a solution of 0.5% collagenase for 30 min at 37°C. Tumour cells were collected from the second digest, washed and cultured overnight at 37°C in RPMI 1640 medium in collagen-covered 24-well plates. After 16 h, the cell adherent layer was washed extensively with RPMI medium and fresh medium was added. Cells were detached from the plates using a solution of 0.25% trypsin in 0.2% EDTA and passaged. Cells were cultured at 37°C in 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mg/ml gentamycin and 50 μM β-mercaptoethanol.

For experiments, cells were washed twice with phosphate buffered saline, pH 7.0 (PBS), applied on a glass plate, dried and fixed with 10% formaldehyde vapour for 3 min. A solution of the fluorescent carbohydrate probe in PBS (0.3 mg/ml, 50–80 μl) was added, and cells were incubated for 1 h in humidified atmosphere at 20°C, then the stain was washed with water and dried in the air. The binding of glycoconjugate to cells was visually estimated with a Leitz Orthoplan fluorescent microscope (Wetzlar, Germany), the 'fluorescein' filter set (495 nm on exitation and 530 nm on emission) being used. Cells incubated with the PBS aliquot were taken as control.

2.3. Preparation of liposomes

Liposome samples had the same matrix lipids (PC–PI, 8:1, by mol); some formulations contained 10 mol% C_{18}Mrph and/or 2 mol % SiaLe^X-vector (or A_{tri}-vector), and were prepared as follows: aliquots of the stock

solutions of matrix lipids in chloroform–methanol (2:1, v/v), C_{18}Mrph and/or carbohydrate vectors were co-evaporated in a round-bottomed flask on a rotary evaporator at 37°C and then dried at 20 Pa for 2 h. Resulting lipid films were dispersed under an inert atmosphere (argon) in PBS to achieve the final matrix lipid concentration of 4.9 mg/ml by ultrasound bath treatment (30 min, 20°C). Suspensions were then extruded five times through polycarbonate membrane filters (Nucleopore, USA) with a pore size of 400, 200 and 100 nm, consecutively. Liposomal average size was 120 nm as determined by a Coulter Model N4 MD Sub-Micron Particle Analyzer (Coulter Electronics, Hialeah, USA). C_{18}Mrph final concentration (0.92 mM) was measured by the ultraviolet (UV) absorption at 260 nm, after 20-fold dilution of a liposomal dispersion aliquot with ethanol. The inclusion of C_{18}Mrph in the liposomes was shown to be complete by gel filtration on a Sephadex G-50 column. The liposomal samples were stored at 4°C and used within 24 h.

2.4. Mice, treatments and tumorigenesis parameters

BLRB males (4.5-month-old) were inoculated subcutaneously (s.c.) near the right flank with the tumour cell suspensions, prepared from syngeneic spontaneous female breast adenocarcinomas according to conventional procedures [23]. Tumour growth was always observed in 100% of the males with grafted tumour cells. Mice of each group (10 animals) were given two intratail vein injections (0.5 ml) of the following formulations on the 3rd and 7th days after tumour cell inoculation: 1, merphalan (0.92 mM, i.e. 140 μg per mouse, approximately 7 mg/kg); 2, liposomes consisting only of the matrix lipids; 3, liposomes with C_{18}Mrph (0.92 mM); 4, liposomes with C_{18}Mrph (0.92 mM) and $SiaLe^X$-vector; 5, liposomes with $SiaLe^X$-vector only; control, PBS. Mice were examined daily for tumour appearance and survival; and weekly three-dimensional size measurements of each tumour with callipers was performed to calculate median tumour diameter using the average of mutually perpendicular parameters: $D = (a + b + h)/3$ where a is maximal length, b is maximal width and h equals the average height of a tumour. Median tumour diameter was found to be an appropriate criterion for the presentation of significant differences amongst the experimental groups, and to be more visual compared with a volume measurement. Based on our previous experience, we consider this method for determining tumour size as reliable. Average time of the tumour appearance and average survival time (AvST) were calculated after the end of the experiment, with the observation time not being limited. The data on relative average tumour growth (RAvTG) and relative average survival time (RAvST) were calculated according to:

$$\frac{\text{parameter in experiment} - \text{parameter in control}}{\text{parameter in control}} \times 100\%.$$

All animal experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (US Department of Health and Human Services, National Health Publication No. 93-23, revised 1985). Each mouse had its individual mark and protocol listing for its whole life and was considered as a patient. The significance of data obtained was determined by the parametric Student *t*-test.

3. Results

3.1. Probing of carbohydrate specificity of tumour cells

To find an effective carbohydrate vector for cytotoxic liposomes designed for the treatment of BLRB males with grafted mammary adenocarcinomas, we have produced primary cell cultures from the tumour and established a cell line. Testing of 34 fluorescent carbohydrate probes for binding with the cells revealed tetrasaccharide $SiaLe^X$ as the ligand with the greatest affinity. Two other probes bearing A_{tri} [GalNAcα1-3-(Fucα1-2)Galβ] and B-trisaccharide (B_{tri}) [Galcα1-3-(Fucα1-2)Galβ] also demonstrated a high binding rate, but this was less than with $SiaLe^X$. The remaining probes containing the following structures displayed low or no binding: α-D-Glc, β-D-Glc, β-D-Gal, α-D-Fuc, α-L-Rha, α-D-Man, β-D-Man-6-phosphate, β-D-Gal-3-sulphate, α-GalNAc, β-GalNAc, β-GlcNAc, β-D-GlcNAc-6-sulphate, α-Neu5Ac, Galβ1-3GalNAcβ, GlcNAcβ1-4GlcNAc, Galα1-3GalNAcα, LacNAc-6-sulphate, GlcNAcα1-3Gal, Galα1-3Gal, Neu5Acα2-6-GalNAcα, $(Neu5Ac\alpha2\text{-}8)_2$, $(Neu5Ac\alpha2\text{-}8)_3$, Fucα1-2-Galβ1-3GlcNAc (H type 1), Neu5Acα2-3Galβ1-4Glc, Neu5Acα2-6Galβ1-4Glc, Fucα1-4(Galβ1-3)GlcNAc (Le^a), Fucα1-3(Galβ1-4)GlcNAc (Le^x), $3'HSO_3Le^a$, $3'HSO_3Le^x$, Fucα1-2Galβ1-4(Fucα1-3)GlcNAc (Le^y) and $SiaLe^a$.

3.2. Preparation of vectored cytotoxic liposomes

Recently we have developed a method for the synthesis of lipophilic glycoconjugates which are suitable for incorporation in the liposomal membrane, i.e. are vectors for liposomes [21]. $SiaLe^X$- (Fig. 1) and A_{tri}-vectors as conjugates with PEG-containing detergent were synthesised in this study. Different liposomal formulations based on the natural lipids, PC and PI, with the lipophilic prodrug C_{18}Mrph and carbohydrate vectors inserted into their bilayer were prepared. Fig. 2 shows a sketch of C_{18}Mrph-loaded carbohydrate-vectored liposome.

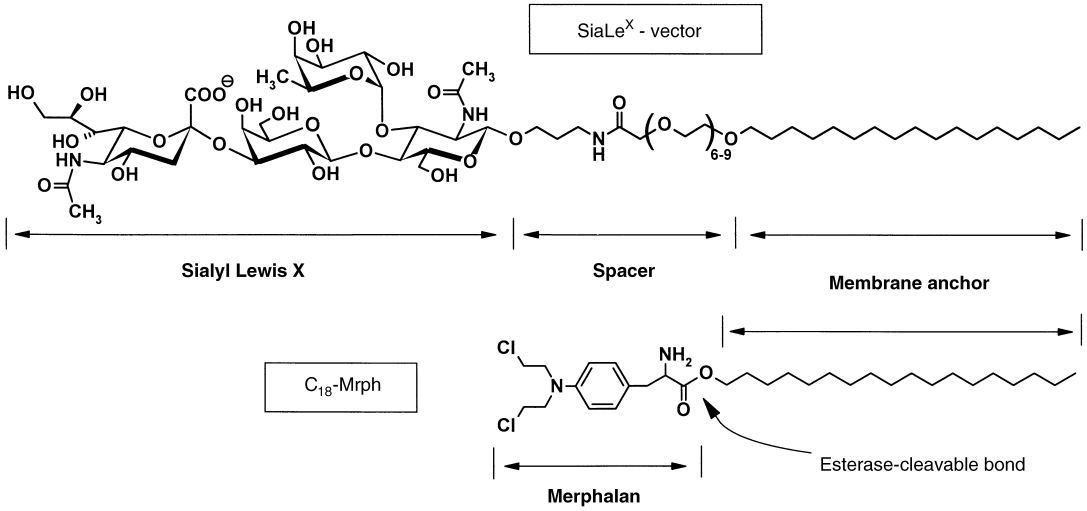

Fig. 1. Molecular structures of SiaLe^X-vector and C_{18}Mrph prodrug.

3.3. Antitumour activity

Six groups of BLRB males with grafted adenocarcinomas were treated with different preparations under the same protocol in order to evaluate the contribution of each component of the formulation. Molar concentrations of the cytotoxic agent merphalan or its lipid derivative C_{18}Mrph and SiaLe^X-vector (if present) in different formulations were equal (Table 1), as were the concentrations of the matrix lipids. Merphalan dose (7 mg/kg) and the schedule of injections were adopted from previous studies [22,23]. Data on the dynamics of the tumorigenesis parameters (the weekly average tumour diameter growth, percentage of animals with no sign of tumour, RAvTG, RAvST and the weekly survival dynamics) for the different experimental groups of BLRB males are shown in Tables 1 and 2 and Fig. 3. Merphalan *per se* (group 1) caused a statistically significant decrease in tumour appearance until week 6 (Tables 1 and 2), and a 1-week delay in the average tumour growth time compared with the control mice. These events led to a pronounced improvement of survival dynamics (Fig. 3) and an average survival time (RAvST = +34%) (Table 2). Empty liposomes (group 2) revealed a weak and insignificant decrease of the tumour growth rate (RAvTG was approximately −15% from week 4 to week 8); and some improvement in survival (RAvST = +16%) (Table 2). Mice treated with liposomal C_{18}Mrph (group 3) showed a substantial delay in tumour appearance compared with control mice (4.7 ± 0.8, and 1.7 ± 0.7 week, accordingly), a statistically significant fall in the tumour growth rate, and a pronounced increase in survival (RAvST = +66%), not only compared with control mice, but also with the animals treated with merphalan itself (group 1) (Tables 1 and 2 and Fig. 3). Further changes in the dynamics of all tumorigenesis parameters were observed for BLRB

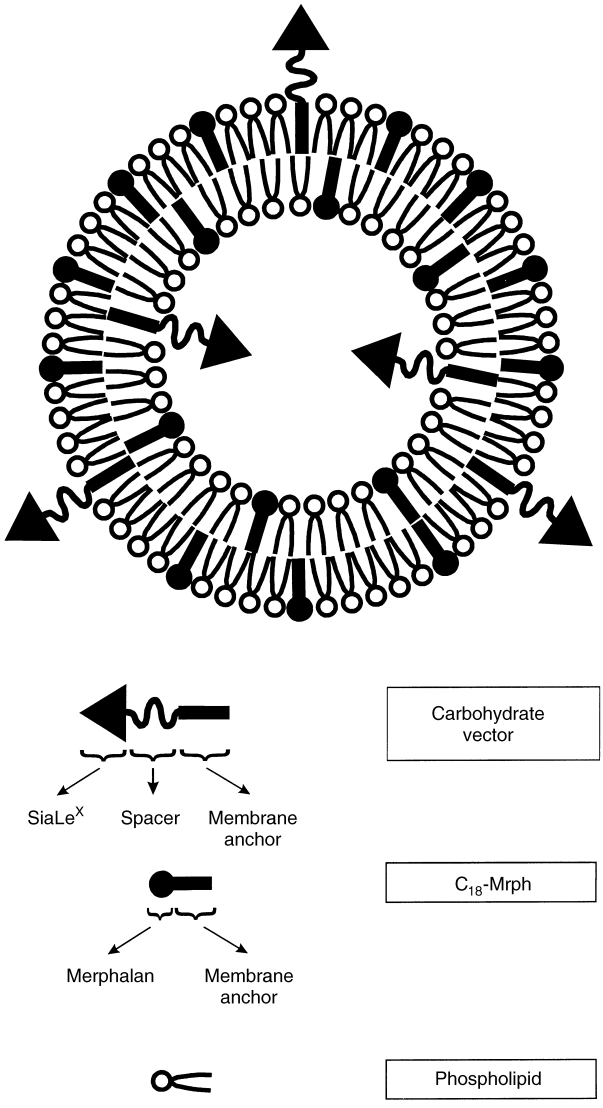

Fig. 2. Schematic structure of a drug-loaded carbohydrate-vectored liposome.

Table 1
Weekly dynamics of averaged median tumour diameter (mm), and percentage of animals with absence of palpable tumours (in parentheses) for different experimental groups[a] of BLRB males[b]

Week	Group 1	Group 2	Group 3	Group 4	Group 5	Control
2	0.9† ± 0.4 (40)	3.4 ± 0.7 (10)	0.1‡ ± 0.1 (90)	0.3† ± 0.3 (90)	2.1 ± 0.4 (10)	3.3 ± 1.0 (10)
3	2.8† ± 0.7 (20)	6.4 ± 1.2	0.2 ± 0.2 (90)	0.3‡ ± 0.2 (90)	4.3 ± 0.7	6.1 ± 1.2
4	7.5‡ ± 1.1	10.9 ± 1.3	1.2§ ± 0.6 (40)	0.1§ ± 0.1 (90)	10.5 ± 1.3	13.0 ± 1.3
5	11.5† ± 1.4	14.1 ± 1.2	4.3§ ± 0.8 (30)	0.9* ± 0.5 (80)	13.2 ± 1.4	16.7 ± 1.4
6	16.2 ± 1.5	17.7 ± 1.2	7.5§ ± 1.2 (20)	1.9* ± 1.3 (80)	18.2 ± 1.6	20.5 ± 1.5
7	18.5 ± 1.3	18.7 ± 1.5	10.7§ ± 2.0 (10)	3.0* ± 1.7 (70)	22.8 ± 1.3	22.0 ± 2.1
8	20.4 ± 1.2	21.0 ± 1.4	14.4§ ± 2.0	4.7* ± 2.3 (50)	25.1 ± 2.1	24.1 ± 2.3
9	21.3 ± 1.0	22.7 ± 1.1	16.2 ± 2.1	5.3* ± 3.0 (50)	–	–
10	21.0 ± 1.2	23.3 ± 0.9	18.0 ± 2.3	6.7* ± 3.3 (50)	–	–

[a] Mice of each group were given two intravenous (i.v.) injections of the following formulations on the 3rd and 7th days after tumour cell inoculation: 1, merphalan (7 mg/kg); 2, liposomes of matrix lipids only; 3, liposomes with C_{18}Mrph (7 mg/kg of merphalan); 4, the same liposomes as in 3 with SiaLe^X-vector; 5, liposomes with SiaLe^X-vector only; control, PBS.

[b] ± Standard error of the mean (SEM). Differences from control group: † $P < 0.05$; ‡ $P < 0.01$; § $P < 0.001$ by Student t-test.

*Differences from group 3. $P < 0.01$ by Student t-test — less than four mice in a group.

Table 2
Weekly dynamics of relative averaged median tumour diameter (%) and relative average survival time (%, in parentheses) for different experimental groups of BLRB males[a]

Week	Group 1	Group 2	Group 3	Group 4		Group 5
				All	'Advanced subgroup'	
2	−73[b]	+3	−97[c]	−91[b]	−89[b]	−36
3	−54[b]	+6	−97[c]	−95[c]	−97[b]	−30
4	−42[c]	−15	−91[d]	−99[d]	−100*	−19
5	−31[b]	−15	−74[d]	−95*	−100*	−21
6	−21	−14	−63[d]	−91*	−100*	−11
7	−16	−15	−51[d]	−86*	−100*	+4
8	−15 (+34)[b]	−12 (+16)	−40[d] (+66)[c]	80* (+137)[d]	−100* (+198)*	+4 (+9)

[a] ± Standard error of the mean (SEM). Differences from control group: [b], $P < 0.05$; [c], $P < 0.01$; [d], $P < 0.001$ by Student t-test.

*Differences from group 3. $P < 0.01$ by Student t-test. For group descriptions see Table 1.

males of group 4 which were treated with C_{18}Mrph-loaded SiaLe^X-vectored liposomes. These mice demonstrated the most impressive fall in terms of tumour appearance and tumour growth rate, with a high statistical significance being observed compared with both the controls and the animals in group 3 (Tables 1 and 2). Average time of the detection of palpable tumours in group 4 exceeded 2-fold the value of this parameter observed for mice in group 3 (10.1 ± 1.9 and 4.7 ± 0.8 weeks, accordingly). Moreover, half of the mice (hereafter called 'advanced subgroup') treated with vectored cytotoxic liposomes attained an average time for the detection of palpable tumours of 15.2 ± 1.7 weeks ($P < 0.001$). The survival in group 4 was dramatically improved compared with the survival obtained for all other groups (Fig. 3). AvST in group 4 was 19.3 ± 2.2 weeks compared with 13.5 ± 1.1 weeks ($P < 0.05$) for group 3. Noteworthy is the AvST for mice of the advanced subgroup: 24.3 ± 2.6 weeks ($P < 0.01$), whilst for the other half of group 4 this value was 14.3 ± 1.5 weeks. RAvST in the whole group was more than 2-fold higher than in group 3, and in the advanced subgroup it tripled (Table 2). Finally, vectored liposomes without cytotoxic agent (group 5) as well as unvectored ones (group 2) caused slight and statistically insignificant inhibition of the tumour growth (Tables 1 and 2).

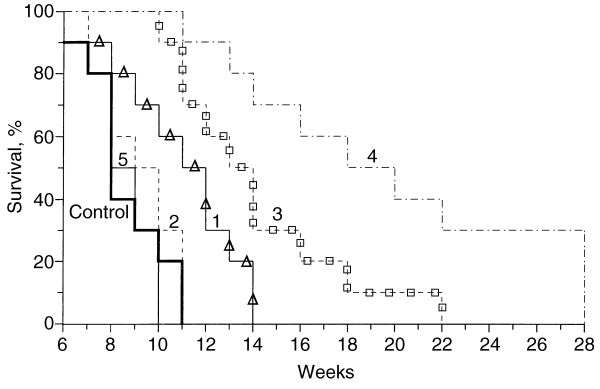

Fig. 3. Weekly survival dynamics for different experimental groups of BLRB males (see footnote to Table 1).

However, survival in these groups was somewhat different (Fig. 3), with group 5 faring slightly worse off compared with group 2 and, at later times, also faring worse than the control mice (Table 2).

A_{tri}-vectored C_{18}Mrph-loaded liposomes did not show a statistically significant improvement in therapeutic efficacy compared with the unvectored cytotoxic liposomes (data not shown).

4. Discussion

Carbohydrate-assisted targeting of drugs to tumours requires first of all the determination of the lectin specificity of tumour cells. Therefore, at the beginning of the study we had to establish a cell line produced from the BLRB mice tumours.

BLRB mouse strain with a high incidence of spontaneous mammary adenocarcinomas in bred and virgin females, caused by the mouse mammary tumour virus is similar to the well known strain C3H and was proposed for the testing of synthetic and natural agents with potential anticancer activity [23,27]. We consider this spontaneous breast cancer mouse model as different from the conventional transplanted ones, where cells of standard mouse or human tumour lines are grafted into mice of a low tumour genotype strain or athymic nude mice. In both cases, tumour antigens are recognisable by antitumour host immune cells. Instead, in our model, mice with a high mammary tumour genotype are used. These mammary tumours have emerged as a result of a long chain of organism/retroviral events. They demonstrate strong unresponsiveness, and both thymic and peripheral deletions of the tumour-reactive T cells [28,29]. We assume these spontaneous mammary tumour mouse models reflect more appropriately the situation with heredofamilial forms of breast cancer in women, and provide additional information for the creation of the whole picture concerning the efficiacy of the testing agent on the tumour-bearing organism. Besides, we have overcome the common difficulties associated with experimental spontaneous tumours, by dealing with initial generations of tumours transplanted into syngeneic males. Such generations are well known to most closely relate to spontaneous cancers.

The next step of the investigation was determination of the tumour lectin specificity. It is known that the highly affinitive carbohydrate–lectin interaction takes place only when both participants are multivalent [14]. This was a reason why we used multivalent polyacrylamide-based fluorescent probes containing a few dozen carbohydrate ligands per carrier molecule. Amongst a wide number of probes tested, $SiaLe^X$ was chosen as the ligand with the greatest affinity. The synthesis of a $SiaLe^X$-bearing vector suitable for the insertion in liposomes was thus carried out. Liposomes also provide the conditions for multivalent interactions allowing single-vector molecules to concentrate by lateral diffusion onto its surface at the site of contact with the receptor molecule of the target cell. Additionally, to enable contact with the receptor molecule, the ligands should be exposed at a sufficient distance over the liposome membrane [30]. Consequently, the molecule of the liposomal carbohydrate vector should be designed as a conjugate of carbohydrate and lipophilic anchor tethered with a hydrophilic spacer. The latter in our case is a short PEG chain (Fig. 1). Earlier Zalipsky and colleagues [31] synthesised $SiaLe^X$ conjugate containing distearoylphosphatidylethanolamine as a lipid anchor and a 2000 D-PEG spacer (approximately 6-fold longer than our PEG chain). This conjugate was shown to incorporate well into preformed PEG-2000 stabilised (Stealth®) liposomes with positioning of the $SiaLe^X$ ligand on the periphery of the outer surface-grafted polymeric 'brush' [31].

Composition of the liposome matrix lipids was stipulated by the following reasons. Inexpensive egg phosphatidylcholine was chosen as the major lipid because it facilitated the preparation of different liposomes owing to its high fluidity. Liposomes bearing a polar drug in the water interior should be prepared from rigid lipids, e.g. distearoylphosphatidylcholine–cholesterol mixtures, in order to prevent leakage of the drug into the circulation (see, for example [12]). This was not needed in our study since the lipophilic prodrug is a membrane component of liposome. Phosphatidylinositol presumably facilitates the incorporation of C_{18}Mrph into the liposome bilayer due to their similar structural shapes and charges. Moreover, the polar headgroups of phosphatidylinositol on the membrane surface, in the concentration being used (approximatley 10 mol%), as has already been shown, provide additional steric stabilisation of the bilayer; this prolongs the circulation time of liposomes and enhances their uptake by tumour [3,6].

Our results testing different liposomal formulations for use in the treatment of mouse mammary adenocarcinoma show that the therapeutic efficacy of merphalan increases significantly after its insertion as C_{18}Mrph into the liposomal lipid bilayer: a delay in tumour incidence and reduction in tumour growth were observed (Tables 1 and 2, groups 1 and 3), with a pronounced improvement in survival (Table 2, Fig. 3). The contribution of empty liposomes to these effects should be negligible (compare with the data obtained for group 2). The most sharp tumour growth inhibition and lifespan prolongation was observed for mice treated with $SiaLe^X$-vectored C_{18}Mrph-loaded liposomes (group 4). Survival in this group was better than in the other groups (Table 2, Fig. 3), with four animals being alive for approximately 6 months. Of note, is the statistically significant difference in the dynamics of tumour growth between groups 4 and 3 (Table 1). These data, together

with the absence of the essential influence of glycoconjugate itself on the antitumour properties of liposomes (compare groups 5 and 2), we consider make a strong argument for the benefit of targeting using cytotoxic liposomes with carbohydrate vectors.

Another explanation for the enhanced antitumour activity of SiaLe^X-vectored C_{18}Mrph-loaded liposomes should also be considered. The tetrasaccharide moiety, as well as the small PEG chains originated from Lubrol PX could produce a hydrophilic steric shield on the membrane surface and, thereby, stabilise the cytotoxic liposomes. This could increase their circulation times resulting in a slow drug release or increased uptake of liposomes by the tumour. However, a previous study has shown [32] that to provide a reliable surface hydrated barrier and prevent opsonisation with such a short PEG chain (of approximately 350 molecular weight) more than 10 mol% of oligomeric moieties should be grafted to the outer leaflet of the liposomal lipid bilayer, a 2 mol% addition being ineffective. In contrast, in the case of long PEG-2000 chains this quantity decreased to 2 mol% [32]. Thus, it seems unlikely that the Lubrol PX hydrophilic spacer itself could provide surface stabilisation in the concentration it was used in this study (2 mol%). Certainly, the addition of the oligosaccharide moiety (SiaLe^X) to the molecular structure of the membrane grafted chains enhances their hydration capacity, which might increase the stability of the liposomes in the circulation. Nevertheless, the equipment of cytotoxic liposomes with another carbohydrate determinant (A_{tri}), which is only one monosaccharide unit shorter than SiaLe^X, did not result in a statistically reliable effect on tumorigenesis parameters compared with unvectored liposomes (data not shown). This fact strongly contradicts the assumption of non-specific steric stabilisation of the carbohydrate-vectored liposomes as a cause of their increased antitumour activity.

Tetrasaccharide SiaLe^X is a low affinity ($K_{dis} \sim 1$ mM) ligand for all three known selectins (E, P and L), being a component of the glycoprotein receptors of these lectins [33]. Tumour cells are known to overexpress selectins which are assumed to play a role in intercellular adhesion and, in particular, in metastasis formation [13]. The results of the present study evidently show that the antitumour activity of the cytotoxic liposomes increases significantly when they are vectored by SiaLe^X, which appeared to be the ligand with the most affinity for the cells of mouse mammary adenocarcinomas. If this effect is a consequence of the direct interaction of liposomes with tumour cells, a question arises: why SiaLe^X-vectored liposomes are not intercepted by selectins of endothelial and blood cells on their route to a tumour? We can argue that the SiaLe^X/selectin interaction mediates the very first stage of the intercellular interaction, rolling; the stage of the following attachment (arrest) requires switching to the next, integrin-mediated mechanisms [33]. Apparently, in the case of SiaLe^X-liposomes rolling-like interaction with endothelial cells is possible. However, the further analogy with the intercellular processes is not the case: liposomes bear no integrin. So, there is no reason to suppose that the SiaLe^X-vector can deliver liposomes to normal cells; moreover, rolling-like interactions with the endothelial cells, which is realised along the gradient of selectin concentration, may be the second factor (together with the high affinity to the adenocarcinoma cells) facilitating the uptake of vectored liposomes by the tumour cells. This hypothesis agrees with the absence of a statistically significant antitumour effect of another carbohydrate vector, A_{tri}, which has relatively high affinity for the tumour cells but cannot mediate rolling.

We therefore conclude that the results obtained testify to the significance and prospects of further investigation of carbohydrate-assisted targeting of drug-loaded liposomes to tumours, with studies of metastases formation, biodistribution, etc. to be performed.

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