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Conjugation of an Anti-B-cell Lymphoma Monoclonal Antibody, LL2, to Long-circulating Drug-carrier Lipid Emulsions

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Abstract

Long-circulating submicron lipid emulsions, stabilized with poly(ethylene glycol)-modified phosphatidylethanolamine (PEG–PE), are promising drug carriers with substantial capacity for solubilization of lipophilic anticancer agents. This study describes the conjugation of the anti-B-cell lymphoma monoclonal antibody LL2 to the surface of lipid-emulsion globules by use of a novel poly(ethylene glycol)-based heterobifunctional coupling agent.

The efficiency of coupling of LL2 to the lipid emulsion was 85% (approx.) and essentially independent of the LL2/emulsion particle ratio and amount of surface-bound PEG–PE. Results from sucrose-gradient centrifugation and Sepharose CL-4B gel filtration indicated stable binding of the antibody to the emulsion. The immunoreactivity of the emulsion–LL2 conjugates was tested with alkaline phosphatase-conjugated LL2 against a monoclonal anti-idiotype antibody, WN. The binding of the conjugates to WN increased with increasing surface density of LL2 up to 40 monoclonal antibodies/emulsion particle, and exceeded that for the free monoclonal antibody (approx. 20 molecules/particle). Results from competitive-binding ELISA were indicative of similar displacement curves for free LL2 and emulsion–LL2 conjugates. Direct cellular ELISA revealed similar binding of emulsion–LL2 complexes to three types of Burkitt's lymphoma cell lines, Raji, Ramos and Daudi.

The results from this study indicate that emulsion–LL2 complexes might be a useful drug-carrier system for more specific delivery of anticancer drugs to B-cell malignancy.

Antibody-based targeting of antineoplastic drugs is a promising approach to the development of therapy for cancer (Vingerhoeds et al 1994; Vitetta & Uhr 1994). Haematological malignancy, in which the immunoconjugates have unrestricted access to cancer cells, should be particularly amenable to this approach.

LL2 is a murine IgG2a anti-B-cell lymphoma monoclonal antibody raised against the Raji Burkitt lymphoma cell line (Pawlak-Byczkowska et al 1989). It has been proven of clinical importance in the radioimmunodetection and radioimmunotherapy of non-Hodgkin's B-cell lymphoma (Goldenberg et al 1991; Murthy et al 1992). That LL2 is internalized into antigen-bearing cells (Shih et al 1994) makes it potentially useful for intracellular delivery of cytotoxic agents. In this study LL2 has been conjugated to the surface of submicron lipid-emulsion drug carriers by use of a heterobifunctional coupling agent.

Submicron lipid emulsions have many appealing properties as drug carriers—they are biocompatible, biodegradable and easy to prepare and handle (Lundberg 1993; Wheeler et al 1994; Liu & Liu 1995; Lundberg et al 1996). The emulsions comprise two major components, an oil core (i.e. triglyceride) stabilized by emulsifiers (i.e. phospholipids). The hydrophobic cores of such emulsions can solubilize considerable amounts of lipophilic drugs (Lundberg 1994, 1997, 1998). The poor emulsifying properties of phospholipids can be enhanced by adding a biocompatible coemulsifier such as polysorbate 80 (Lundberg 1994). A severe problem with particulate drug carriers is rapid uptake by the mononuclear phagocyte system (Poste et al 1982). Although this problem can be

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overcome by engrafting poly(ethylene glycol) (PEG) chains on to the particle surfaces (Woodle & Lasic 1992; Lundberg et al 1996), higher molecular-weight PEGs mask antibody recognition and weaken the targeting capability of immunoconjugates (Blume et al 1993). A successful strategy for solving this problem is to link the monoclonal antibody to the distal PEG terminus (Song et al 1996; Kirpotin et al 1997; Lopes de Menezes et al 1998). An obvious problem with particulate drug carriers such as liposomes and emulsions is slow and non-specific uptake by cells. The conjugation, to the surface of the drug carrier, of an internalizing ligand for cellular receptors would greatly enhance the feasibility of this concept of drug delivery. This paper reports the successful conjugation of an internalizing ligand, monoclonal antibody LL2, to a long-circulating submicron lipid emulsion by means of a heterobifunctional coupling agent.

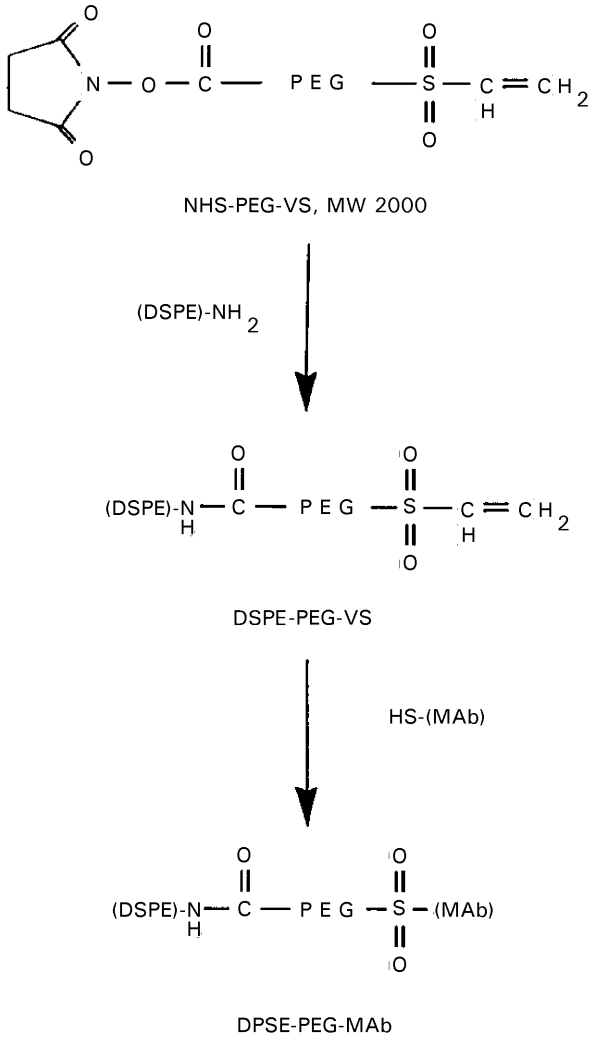

Figure 1. Synthesis of a PEG–DSPE derivative with a vinylsulphone (VS) function at the end of the poly(ethylene glycol) chain, and its reaction with thiol groups on a monoclonal antibody.

Materials and Methods

Materials

Dipalmitoylphosphatidylcholine (DPPC) was obtained from Alexis (Läufelfingen, Switzerland). Dipalmitoylphosphatidylethanolamine (DPPE), distearoylphosphatidylethanolamine, triolein, polyoxyethylenesorbitan monooleate (polysorbate 80), carbonyl diimidazole, maleimide-activated alkaline phosphatase, and 2-mercaptoethanol were from Sigma (St Louis, MO). Poly(ethylene glycol)vinylsulphone-*N*-hydroxysuccinimidyl ester (VS–PEG–NHS) was purchased from Shearwater Polymers Europe (Enschede, The Netherlands). [^{14}C]DPPC and ^{125}I were from Amersham International (Amersham, UK). 1,11-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was from Molecular Probes (Eugene, OR) and 4-methylumbelliferyl phosphate (MUP) from Koch–Light (Suffolk, UK). Poly(ethylene glycol)-modified phosphatidylethanolamine (PEG–PE) was synthesized by reaction of PEG 2000 with carbonyldiimidazole then addition of DPPE (Allen et al 1991). Finally PEG–DPPE was dialysed against water by means of a Spectra/Por CE 300 000 MWCO dialysis bag (Spectrum Medical Industries, Houston, TX) and then lyophilized (Maruyama et al 1992). A poly(ethylene glycol) derivative of phosphatidylethanolamine with a vinylsulphone group at the distal terminus of the poly(ethylene glycol) chain (DSPE–PEG–VS) (Figure 1) was synthesized by reacting NHS–PEG–VS (25 μmol) with DSPE (23 μmol) and triethylamine (50 μmol) in chloroform (1 mL) for 6 h at 40°C. The product was purified by preparative silica gel thin-layer chromatography (TLC) and gave a single spot in analytical TLC when visualized with iodine vapour and molybdate spray.

Cell lines and monoclonal antibody

Cultured cells from man included Burkitt's lymphoma lines Raji, Daudi and Ramos. The cells were grown in RPMI 1640 medium with 10% foetal calf serum; they were maintained at 37°C and aerated with 5% CO_2 in air.

Murine monoclonal antibody LL2, an I7G (Pawlak-Byczkowska et al 1989) specific for the epitope B of CD22 (Stein et al 1993) raised by immunization of BALB/c mice with an extract of Raji cells, and WN, a rat monoclonal anti-idiotype

antibody to LL2 (Losman et al 1995) were obtained from Immunomedics. The molecular weight of LL2 was found to be 154 kD by mass-analysed laser-desorption ionization–time-of-flight (MALDI–TOF) mass spectrometry (Mass Consortium, San Diego, CA). ^{125}I labelling was performed by the chloramine-T procedure. For alkaline phosphatase conjugation of LL2, the monoclonal antibody was first reduced with 50 mM 2-mercaptoethanol for 10 min at 4°C in 0·2 M Tris buffer (pH 8·7) and then reacted with maleimide-activated phosphatase (2 mol enzyme (mol LL2)$^{-1}$) in 0·15 M NaCl and 0·1 M sodium phosphate (pH 7·0) for 3 h at room temperature. Remaining maleimide groups were blocked with 1·5 mM 2-mercaptoethanol.

Preparation of emulsion

Submicron lipid emulsions were prepared and characterized as described in detail elsewhere (Lundberg 1993; Lundberg et al 1996). The basic composition of the lipid emulsions was triolein–DPPC–polysorbate 80 2 : 1 : 0·4 (w/w). When indicated, 2–8 mol% PEG–DPPE (calculated relative to DPPC) was added into the lipid mixture. For antibody conjugation, 2 mol% DSPE–PEG–VS (calculated relative to DPPC) was included. The components were dispensed into vials from stock solutions at −20°C and the solvent was evaporated to dryness under reduced pressure. Phosphate-buffered saline (PBS) was added and the mixture was heated to 50°C, vortex mixed for 30 s, and sonicated with an MSE probe sonicator for 1 min. Emulsions were stored at 4°C and used for conjugation within 1 day.

Conjugation of LL2 to lipid emulsions

Coupling of LL2 to emulsion globules was performed by reaction of the vinylsulphone group at the distal PEG terminus on the surface of the globules with the free thiol groups on the monoclonal antibody (Figure 1). Before conjugation LL2 was reduced by reaction with 2-mercaptoethanol (50 mM) for 10 min at 4°C in Tris buffer (pH 8·7; 0·2 M). The reduced monoclonal antibody was separated from excess 2-mercaptoethanol by use of Sephadex G-25 spin-columns, equilibrated in sodium acetate (50 mM)-buffered 0·9% saline (pH 5·3). After this procedure the antibody exposed 8–10 free thiol groups. The product was assayed for protein concentration by measurement of its absorbance at 280 nm (it was assumed that a 1 mg mL^{-1} monoclonal antibody solution has an absorbance of 1·4) or by quantitation of ^{125}I-labelled monoclonal antibody. Thiol groups were determined with Aldrithiol, by monitoring the change in absorbance at 343 nm, with cysteine as standard. The coupling reaction was performed overnight, at room temperature under argon, in HEPES-buffered saline (pH 7·4). Excess vinylsulphone groups were quenched with 2 mM 2-mercaptoethanol for 30 min (Kirpotin et al 1997), excess 2-mercaptoethanol and antibody were removed by gel chromatography on a Sepharose CL-4B column. The immunoconjugates were collected near the void volume of the column, passed through a 0·45-μm sterile filter, and stored at 4°C. Coupling efficiency was calculated by use of ^{125}I-labelled monoclonal antibody. Recovery of emulsions was estimated by measurement of [^{14}C]DPPC in parallel experiments. The number of monoclonal antibody per emulsion globule was calculated assuming a droplet size of 50 nm (Lundberg 1993) and a molecular weight of 150 kD for LL2.

The integrity of the emulsion–LL2 conjugates was checked by sucrose density-gradient centrifugation and gel filtration. The protein fraction was quantified by ^{125}I counting and the emulsion by DiI fluorescence (excitation and emission wavelengths 520 and 578 nm, respectively). Centrifugation (50 000 rev min^{-1}, 24 h at 10°C) was performed in a Beckman SW-60 Ti rotor. After centrifugation the tubes were punctured and fractions were collected. Gel filtration was performed on a 30 cm × 2 cm Sepharose CL-4B column eluted with PBS (0·25 mL min^{-1}).

ELISA assay and cell binding

The immunoreactivity of the emulsion–LL2 conjugates was assessed by determining their binding affinities to the anti-idiotype antibody WN IgG. ELISA plates were coated with WN, 50 μL/well of a solution (10 μg mL^{-1}) in carbonate buffer (pH 9·6; 50 mM), at 4°C overnight. The wells were washed with 1% Triton X-100 in PBS and additional binding sites blocked with dilution buffer containing 1% bovine serum albumin (BSA) and 1% Triton X-100 in PBS. Alkaline phosphatase-conjugated free and emulsion-associated LL2 with different monoclonal antibody/emulsion particle ratios in dilution buffer were then added to the plates. After incubation for 1 h at room temperature, unbound antibody was removed by washing three times with 1% BSA in PBS. Binding of alkaline phosphatase-conjugated LL2 was then determined by measuring hydrolysis of MUP (0·1 mg mL^{-1}) in Tris buffer (pH 8·0) by means of a microtitre plate spectrofluorimeter with a 365-nm excitation filter and a 450-nm emission filter. The competitive blocking assays were performed with

the same protocol as above, but the WN-coated wells were incubated with alkaline phosphatase-conjugated free and liposome-associated LL2 in the presence of excess unconjugated LL2 (0·1–10 μg mL^{-1}).

The binding of emulsion–LL2 complexes to target cells was measured by use of alkaline phosphatase-conjugated monoclonal antibody. Cells (2×10^6) in growth medium were incubated with alkaline phosphatase-conjugated LL2 and its emulsion conjugates for 30 min with shaking at 4°C. The cells were thoroughly washed with ice-cold PBS and the alkaline phosphatase activity was measured.

Results and Discussion

Conjugation

A serious problem associated with the use of particulate lipid and lipid–protein drug-carriers is their very rapid elimination from the bloodstream by the cells of the mononuclear phagocyte system. Although the development of long-circulating dose-independent lipid drug-carrier formulations with engrafted PEG on their surface is a major breakthrough in this field (Woodle & Lasic 1992), surface-attached PEG groups have been shown to interfere with the efficient binding of targeted liposomes to their cellular targets (Klibanov et al 1991; Park et al 1995). With liposomes this problem has recently been tackled by attachment of the targeting antibody to the termini of the surface-grafted PEG chains (Kirpotin et al 1997; Lopes de Menezes et al 1998). In this study a similar approach has been applied to long-circulating lipid emulsions. A novel heterobifunctional coupling agent, NHS–PEG–VS, is used for the conjugation process. The coupling procedure depicted in Figure 1 starts with reaction of the NHS ester group of NHS–PEG–VS, with the amino group of DSPE. The NHS ester is highly reactive toward amino groups, but hydrolytically unstable. Under the conditions used, reaction in chloroform in the presence of triethylamine, the yield was very high (approx. 90%). The next step was reaction of the vinylsulphone (VS) with thiol groups on the monoclonal antibody. Vinylsulphone is an attractive derivative for selective coupling to thiol groups. At near neutral pH VS will couple with a half-life of 15–20 min to proteins containing thiol groups (Morpurgo et al 1996). The reactivity of VS is a little lower than that of maleimide, but the VS group is more stable in water and a stable linkage is produced by reaction with thiol groups. The conjugation of reduced LL2 to the VS group of the surface-grafted DSPE–PEG–VS during 16 h at room temperature was very reproducible with an typical efficiency of 85% (approx.) The quenching of excess active groups after the conjugation reaction seems to be important, because in the presence of free thiols or hydrazide groups at the PEG termini immunoliposomes bind appreciably to cells lacking the target antigen (Allen et al 1995; Hansen et al 1995), probably by covalent attachment to cell surface molecules.

Coupling efficiency

To explore the proper conditions for the conjugation process the starting ratio of LL2 to emulsion globules was varied. The results showed that the coupling process was essentially independent of the starting ratio in the range 5–40 monoclonal antibodies per emulsion particle (Figure 2A). Kirpotin

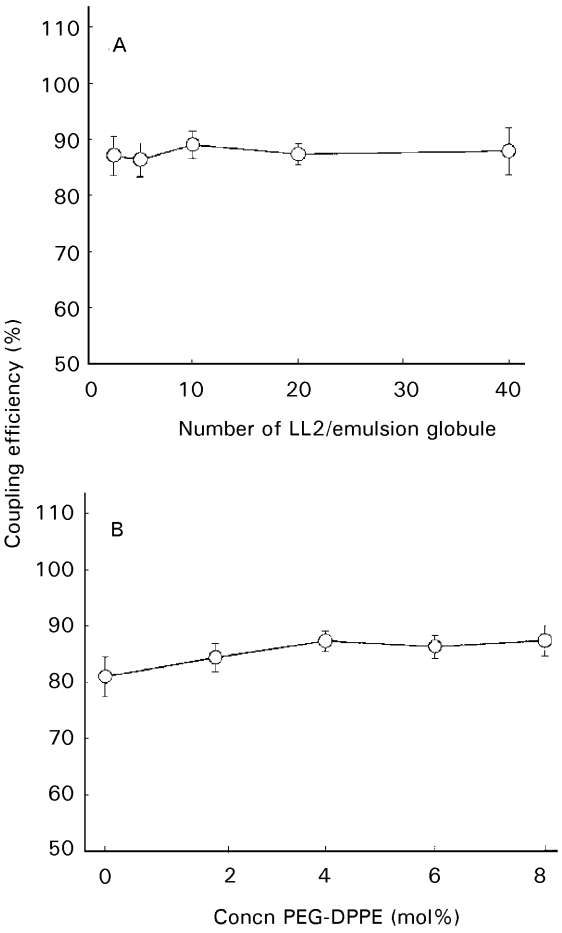

Figure 2. Efficiency of coupling of LL2 to vinyl sulphone-activated lipid emulsions as a function of A. LL2/emulsion particle ratio (4% PEG–DPPE) and B. amount of PEG–DPPE (LL2/emulsion particle ratio 20 : 1).

et al (1997) showed that the number of monoclonal antibodies per liposome could be increased to 100–120 without loss of coupling efficiency, although the amount of total cell-associated immunoliposomes reached a plateau at (approx.) 40 monoclonal antibodies/liposome suggesting that there is no need to increase the amount of liposome-conjugated protein beyond this value. It has been shown that high antibody densities on liposomes induce rapid clearance from plasma (Allen et al 1995). Another factor which had to be considered was the effect of the steric stabilizer PEG–DPPE. It has been shown that surface-grafted PEG reduces the conjugation efficiency of short spacer linkers, especially when the PEG–PE content is increased to 3·5% and more, corresponding to the transition from ''mushroom'' to ''brush'' conformation of the surface-grafted PEG (Kenworthy et al 1995). In this study, with PEG chains of Mr = 2000 for both PEG–DPPE and DSPE–PEG–VS, the coupling was essentially unaffected by PEG–DPPE content (Figure 2B). This result is in agreement with that obtained with long PEG-spacer linkers for the conjugation of monoclonal antibody to liposomes (Kirpotin et al 1997).

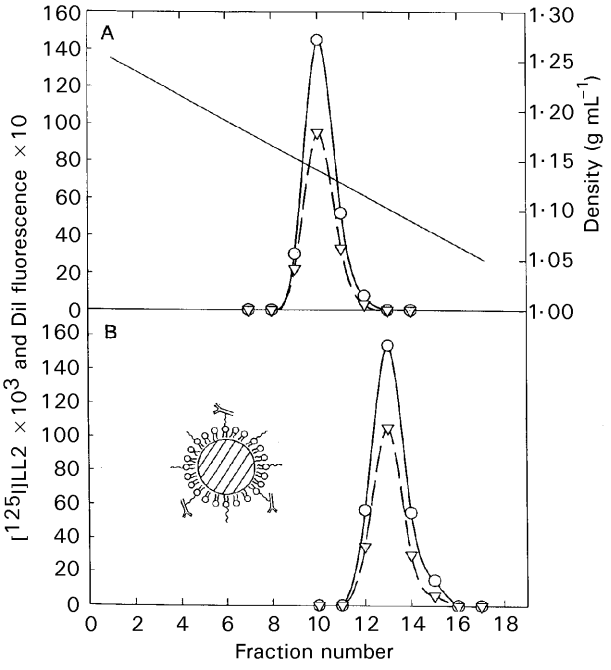

Figure 3. Characterization of lipid emulsion–LL2 complexes by A. sucrose density-gradient centrifugation and B. gel filtration on a Sepharose CL-4B column. LL2 was quantified by measurement of ^{125}I radioactivity (○) and the emulsion core by DiI fluorescence (▽). Insert. Schematic diagram of emulsion–LL2 globule.

Characterization of conjugates

A previous study showed that a combination of phosphatidylcholine and the non-ionic surfactant polysorbate 80 enables the preparation of stable lipid emulsions with a particle diameter of (approx.) 50 nm (Lundberg 1993). Coating of such emulsion particles with PEG-DPPE results in a prolonged circulation time after parenteral administration (Lundberg et al 1996). These submicron lipid emulsions have been shown to be excellent drug carriers for solubilization of lipophilic anticancer drugs (Lundberg 1994, 1997, 1998). The stable association of LL2 with the drug-carrier emulsions was determined by gel chromatography on a Sepharose CL-4B column and sucrose density-gradient centrifugation (Figure 3). The gel filtration profile contained a symmetrical peak where the ^{125}I-labelled protein component coeluted with the emulsion fraction labelled with the lipophilic fluorescence marker DiI. In sucrose density-gradient centrifugation components labelled in the same way appeared as peaks at densities of 1·07 g mL^{-1} (approx.)

Immunoreactivity of LL2–emulsion conjugates

The monoclonal anti-idiotype antibody, WN, to LL2 was used to determine LL2 immunoreactivity. WN is exquisitely specific for LL2 and reacts with no other monoclonal antibody and inhibits the binding of LL2 to Raji cells (Losman et al 1995). The binding values presented in Table 1 clearly show that binding increases with the number of LL2 molecules per emulsion globule and exceeds that for free LL2 at ca 20 monoclonal antibody molecules/globule. The result is, however, obscured because binding of one emulsion-conjugated LL2 results in association with WN of the rest of the monoclonal antibody molecules on the same particle. Despite this complication the

Table 1. Evaluation of the immunoreactivity of emulsion–LL2 conjugates by ELISA.

Monoclonal antibody/emulsion particle ratios	LL2 binding
5 : 1	31·1 ± 3·8
10 : 1	77·5 ± 1·8
20 : 1	112·2 ± 5·9
40 : 1	123·2 ± 3·2

The plates were coated with the anti-idiotype monoclonal antibody, WN, and alkaline phosphatase-conjugated free and emulsion-associated LL2 with different monoclonal antibody/emulsion particle ratios were added to the plates. After 1 h incubation the binding of alkaline phosphatase-conjugated LL2 was measured (free LL2 = 100). Values are means ± standard deviations, n = 4.

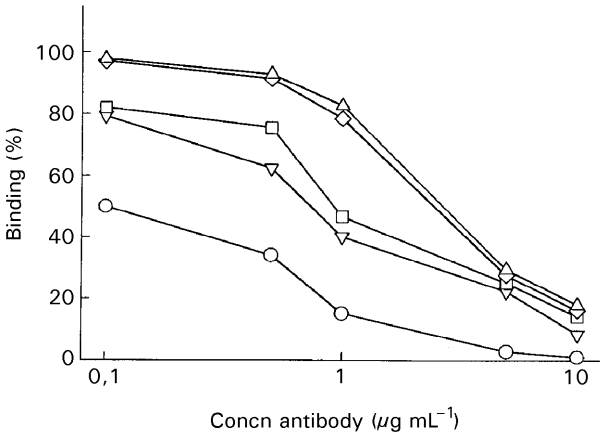

Figure 4. Competitive binding ELISA with WN IgG-coated plates of alkaline phosphatase-conjugated free (△) and emulsion-associated LL2 with monoclonal antibody/emulsion particle ratios of 5 : 1 (○), 10 : 1 (▽), 20 : 1 (□), and 40 : 1 (◇).

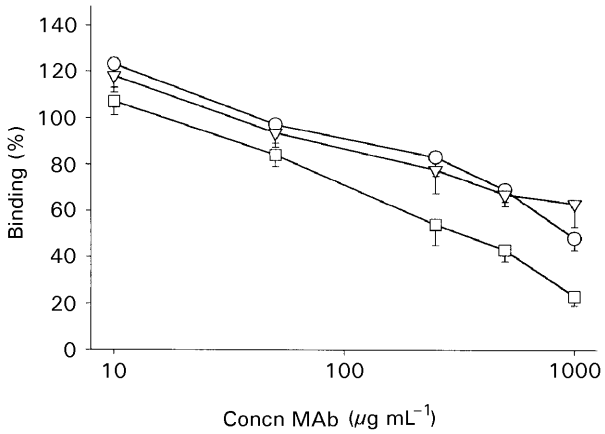

Figure 5. Cell-binding assay of emulsion–alkaline phosphatase-conjugated LL2 complexes (20 monoclonal antibodies/emulsion particle) with Raji (○), Ramos (□), and Daudi (▽) cells. The binding is given as percentage of free LL2.

results strongly indicate that the immunoreactivity of the conjugated monoclonal antibody is preserved. The immunoreactivity of conjugated LL2 was further tested with competitive binding ELISA. The results, presented in Figure 4, show that displacement curves were similar for free and emulsion-bound alkaline phosphatase-conjugated LL2, coincubated with excess free LL2. The immunoreactivity of the emulsion–LL2 conjugates was further verified by a cell-binding assay with alkaline phosphatase-conjugated LL2 preparations. The results presented in Figure 5 show that at low concentrations the binding of LL2–emulsion complexes and free LL2 was similar, but the relative binding affinities of the complexes decreased with increasing concentration.

Previous papers from this laboratory have described the preparation, drug incorporation, antineoplastic activity and in-vivo behaviour of drug-carrier lipid emulsions. This study is a further step towards a more specific delivery system whereby drug-carriers can be supplied with a homing device in the form of a monoclonal antibody against an internalizing antigen on cancer cells. We believe that lipid emulsion–LL2 conjugates are a potential targeting system for more specific delivery of anticancer agents to neoplastic cells.

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