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Specific Targeting of Phototoxic Haptenated Liposomes to a Hapten-Specific B Cell Lymphoma[1]

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A method is reported to eliminate B lymphocytes specific for a haptenated lipid by using the lipid hapten to target a photosensitive drug to them. The photosensitizer eosin was coupled to a phospholipid and incorporated into trinitrophenol (TNP)-bearing small unilamellar vesicles of egg phosphatidylcholine (PC) and cholesterol in order to target the photosensitizer to B lymphoma cells (A20-HL) with TNP-specific membrane IgM receptors *in vitro*. Exposure of the treated cells to visible light led to an antigen-specific toxic effect indicated by inhibition of cell proliferation. A significantly higher concentration of liposomal eosin was required to inhibit control B cells. These were genetically identical B lymphoma cells (A20-2J) which lack only the DNA for the surface antigen receptor. Furthermore, pretreatment with TNP-conjugated keyhole limpet hemocyanin or anti-IgM antibody abolished the antigen-specific toxic effect, confirming that the TNP-targeted liposomal eosin mediates its effect by binding to the Ig antigen receptors on TNP-specific B cells. Incubation of cells with the TNP-bearing phototoxic liposomes at 4°C instead of 37°C did not alter the antigen-specific targeting effect, suggesting that uptake of the liposomal drug into the cells is not necessary for its toxic effect. Replacement of the liposomal phospholipid (egg PC) with saturated species of PC having higher phase transition temperatures or with sphingomyelin caused a decrease of the antigen-specific effect. These results demonstrate the potential use of antigen-bearing liposomal phototoxic drugs for the purpose of targeting and eliminating B cells with antigen-specific surface Ig receptors.

INTRODUCTION

Humoral response is involved in the pathogenesis of a number of autoimmune diseases. Current treatment for these types of diseases includes plasmapheresis, high dose immunoglobulin, or drugs which cause general suppression of the immune system (1, 2). The specific elimination of the self antigen-specific B cell clones without affecting other immune cells would be a preferable treatment.

Enhanced drug selectivity for specific cells can be achieved by using a ligand to deliver drugs to cells which express a membrane receptor for the ligand. Antigens have been used in a number of studies as the ligand to target covalently bound drugs or toxins to specific B cells with Ig receptor for the antigen (3–13).

The self-antigen involved in some autoimmune diseases consists of carbohydrate epitopes found on both glycoproteins and glycolipids (14). For example, a variety of peripheral neuropathies and motor neuron disorders are associated with large amounts of circulating monoclonal or polyclonal antibodies specific for several glycolipids in the peripheral nervous system (15–19). There is evidence that antibody binding to myelin or to peripheral nerve plays an important role in the pathogenesis of these diseases (20–25).

Liposomes are ideal carriers for targeting drugs to carbohydrate-specific B cells because glycolipids can easily be incorporated into the lipid bilayer. Incorporation of the glycolipid in a liposome bilayer ensures that its hydrophilic sugar moiety is properly presented for binding to the carbohydrate-specific B cell Ig receptor. Binding of Ig and other protein receptors to the carbohydrate of glycolipids often depends on how the glycolipid is presented (26–28, 50). Furthermore, liposomes are generally nontoxic, biodegradable, and nonimmunogenic and they are able to accommodate a wide range of cytotoxic compounds in large quantities (29). However, although antigen-specific binding between antigen-bearing liposomes and the antigen-specific surface Ig receptors on B cells has been demonstrated by several investigators (30–32), an antigen-specific cytotoxic effect on the B cells by antigen-bearing liposomal drugs has not yet been achieved. This failure may be due to low internalization of liposomes by the B cells and/or to the use of target B cell lines which do not express

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sufficient amounts of membrane antigen-specific Ig receptor.

Since glycolipid-specific B cell lines with sufficient amounts of membrane receptors are not available, we used the hapten-specific transformed B lymphoma cell line (A20-HL) which expresses the membrane form of IgM specific for trinitrophenol (TNP)[3] (33) as a model target B cell. TNP coupled to phospholipid was used for targeting the liposomes to the B cells. The photosensitizer eosin was used as the liposomal drug since it is cytotoxic without the need to be internalized (34, 35). It was bound covalently to a phospholipid and incorporated into TNP-bearing small unilamellar vesicles (SUVs) in order to minimize drug leakage or diffusion from the liposomes. Recently, other phototoxic agents have been incorporated into antibody-targeted liposomes (36, 37) or bound covalently to membrane fragments of antigen-presenting cells to specifically kill selected cells *in vitro* (38). We show antigen-specific binding and a toxic effect on the TNP-specific B cells by the TNP-targeted eosin-containing liposomes *in vitro*.

MATERIALS AND METHODS

Reagents and Antigens

Trinitrophenylated keyhole limpet hemocyanin (TNP_{30}-KLH) was prepared by the reaction of 2,4,6-trinitrobenzene sulfonic acid with KLH (both reagents purchased from Sigma Chemical Co., St. Louis, MO) at room temperature under alkaline conditions as described by Good *et al.* (39). The product was extensively dialyzed against 0.001 *M* phosphate buffer (pH 7.4) and had approximately 30 mol of TNP per 100,000 g KLH determined as described in (39). Eosin isothiocyanate and 5-(*N*-hexadecanoyl)amino eosin were purchased from Molecular Probes, Inc. (Eugene, OR). Bovine serum albumin (BSA) and L-histidine were purchased from Sigma. Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden). *N*,*N*-Dimethyl-4-nitrosoaniline (RNO) was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI).

Antibodies

Rat anti-mouse IgM (heavy chain) and mouse anti-rat IgG2a were purchased from Serotec Canada (Toronto). Biotin-conjugated rat anti-mouse IgM monoclonal antibody and streptavidin–phycoerythrin conjugate were purchased from Pharmingen (San Diego, CA).

[3] Abbreviations used: TNP, trinitrophenol; SUVs, small unilamellar vesicles; KLH, keyhole limpet hemocyanin; RNO, *N*,*N*-dimethyl-4-nitrosoaniline; PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; SM, sphingomyelin; TNP-DPPE, *N*{[6-(2,4,6-trinitrophenyl) amino] caproyl} dipalmitoyl-L-α, phosphatidylethanolamine; PBS, phosphate-buffered saline; DNP, dinitrophenol; chol, cholesterol.

Lipids

Egg phosphatidylcholine (egg PC), dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), and cholesterol were purchased from Sigma. Dipalmitoylphosphatidylethanolamine (DPPE) was purchased from Calbiochem Corp. (La Jolla, CA). Bovine brain sphingomyelin (SM) and *N*-{[6-(2,4,6-trinitrophenyl) amino] caproyl} dipalmitoyl-L-α-phosphatidyl ethanol amine (TNP-DPPE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). L-*a*-dipalmitoyl (2-palmitoyl-9,10-^{3}H(*N*)-phosphatidylcholine (sp act 58.0 Ci/mmol) (^{3}H-DPPC) was purchased from Dupont NEN Products (Boston, MA). AsialoGM_2 was a kind gift from Dr. C. Lingwood (The Hospital for Sick Children, Toronto, Canada). All lipids were chromatographically pure and were stored at −20°C. The egg PC and asialo GM_2 were stored under nitrogen.

Cell Lines and Culture Conditions

The B lymphoma cell lines A20-2J and A20-HL (a transformed B lymphoma of A20-2J which expresses membrane IgM specific for TNP) (33) were a generous gift from Dr. N. Hozumi (Mount Sinai Hospital, Toronto, Canada). The parent cell line A20-2J has surface IgG but no IgM. The A20-HL line was able to take up and present TNP-KLH to a comparable extent as enriched normal TNP-specific B cells (33). The cells were grown in RPMI 1640 without phenol red (Materials Distribution Centre, University of Toronto) and supplemented with 10% fetal calf serum, 50 m*M* 2-mercaptoethanol, 100 m*M* nonessential amino acids, and 0.1 mg/ml of geneticin (Gibco Laboratories, Grand Island, NY).

Flow Cytometry

The expression of cell surface IgM on the A20-HL and A20-2J B lymphoma cell lines was determined by flow cytometry under the following conditions. Cells (2×10^6) were washed three times with cold PBS containing 3% fetal calf serum (washing medium) and resuspended in 0.1 ml of washing medium containing 2.5 μl of biotinylated anti-IgM antibody for 30 min at 4°C in the dark. After three washes, the cells were stained with 0.1 ml of washing medium containing 0.25 μl of streptavidin–phycoerythrin conjugate for 30 min at 4°C in the dark. After three more washes, the cells were suspended in 1.0 ml of washing medium and analyzed using a FACScan equipped with the Consort 340 system (Hewlett Packard). The negative control consisted of cells treated with streptavidin–phycoerythrin but not preincubated with the biotinylated anti-IgM antibody. The data are presented as histograms of log fluorescence intensity in fluorescence units vs cell frequency.

Synthesis and Purification of Eosin-Conjugated DPPE (Eosin-DPPE)

Eosin-DPPE with the eosin covalently attached to the polar head group of the lipid was prepared from DPPE and the amino-reactive dye, eosin isothiocyanate. In general, 20 mg of DPPE was reacted with 20 mg of dye in 4.0 ml of tetrahydrofuran containing 14% H_2O (to aid in solubilizing the DPPE) and 130 μl of triethylamine, at room temperature in the dark for 1 to 2 hr. The reaction mixture was dried under a stream of nitrogen gas and then under vacuum. The residue was dissolved in chloroform:methanol:H_2O (65:25:4, v/v/v) and the eosin-lipid was purified using a silica gel filtration column equilibrated with the same solvent mixture. Three bright orange-colored bands were followed visually and collected in 12-ml fractions. The first two bands overlapped somewhat and the third band was retained on the column long after the elution of the first two bands. Several fractions of the first two bands as well as the starting materials, eosin isothiocyanate, and DPPE, were examined by thin-layer chromatography (TLC) using 0.25-mm silica gel glass plates (Rose Scientific, Edmonton, Alberta) and 65:25:4 (chloroform:methanol:H_2O, v/v/v/) as the developing solvent. After development, eosin isothiocyanate gave one bright orange spot about midway along the TLC plate and DPPE ran further up as indicated by a single spot detected by copper acetate/phosphoric acid spray. The fractions containing eosin-DPPE were identified by a single orange-colored spot migrating below eosin isothiocyanate which spotted positive with copper acetate/phosphoric acid spray. These fractions were pooled and dried under a stream of nitrogen and then under vacuum. Spectrophotometric analysis of the pooled eosin-DPPE fractions showed a strong absorption band at 528 nm.

Preparation of TNP-Targeted Eosin-Containing (SUVs)

Liposomes were prepared fresh for each experiment by probe sonication using a sonifier cell disruptor (Branson Instruments, Inc., Melville, NY) at 50–60 W. In routine experiments, chloroform:methanol (2:1, v/v) solutions of TNP-DPPE, eosin-DPPE, egg PC (unless otherwise noted), and cholesterol were mixed in a large round-bottom glass tube. The solvent was evaporated under a stream of nitrogen and dried further under vacuum for at least 2 hr. A few glass beads were added to the tube and the lipids were dispersed in sterile 0.1 *M* phosphate-buffered saline (pH 7.4) at 80°C for 10 min with frequent intermittent vortexing. The saline was sterilized by filtration through a Falcon 0.22 μm cellulose acetate membrane (Becton–Dickinson, Lincoln Park, NJ). Typically, the liposome suspension was about 4 m*M* total lipid with TNP-DPPE:eosin-DPPE:eggPC:cholesterol at a mole ratio of 2:5:48:45, respectively. Liposomes containing other phospholipids (SM, DMPC, DPPC, DSPC) were also prepared in the same way. The liposome suspension was sonicated to optical clarity (about 10 min probe sonication) and then centrifuged for 10 min at 10,000 rpm to remove any large multilamellar liposomes. Spectrophotometric analysis of the TNP-targeted eosin-containing SUVs (with or without 10% SDS) showed a strong absorption band at 528 nm.

Gel Filtration Chromatography of TNP-Targeted Eosin-Containing SUVs

A Sepharose 4B column (2.5 × 25 cm) was prepared and equilibrated with PBS (pH 7.4). The column was first saturated with a lipid dispersion containing PC:cholesterol and eosin-DPPE to prevent nonspecific binding of the TNP-bearing eosin-containing PC:cholesterol liposomes to the column, and then washed and equilibrated overnight with PBS (pH 7.4). A 1.0-ml preparation of TNP-targeted eosin-containing SUVs (TNP-DPPE/eosin-DPPE/egg PC/cholesterol mole ratio 2:5:48:45) containing traces of ^{3}H-DPPC was applied and the column was run at approximately 0.2 ml/min. Two orange-colored bands were followed visually and collected in 1.0-ml fractions. The fractions were monitored for their absorption at 528 nm (indicator of eosin-DPPE) and their radioactivity (indicator of liposomal phospholipid). The ratio of absorption:cpm of the liposome preparation, before and after application to the column, was determined. The liposomal fractions were pooled and concentrated using a Centriplus-3 (Amicon) to about a 1.0-ml volume. This was reapplied to the column and the absorption:cpm ratios of the fractions measured to determine any loss of eosin-DPPE from the liposomal bilayers.

Singlet Oxygen Production by TNP-Targeted Eosin-Containing Liposomes

The production of singlet oxygen by TNP-targeted eosin-containing liposomes was measured by the method of Kraljic and El Mohsni (40). This method is based on the bleaching of RNO in the presence of histidine. The light activation of the photosensitizer results in the production of singlet oxygen molecules which react with histidine to form a *trans*-annular peroxide intermediate responsible for the decrease in the absorbance of RNO at 440 nm. Mixtures of TNP-targeted eosin-containing SUVs (TNP-DPPE, 5-(*N*-hexadecanoyl)amino eosin, and DMPC in a mole ratio of 2:3:95, respectively), 10 m*M* histidine, and 30 μM RNO in 0.01 *M* PBS (pH 7.4) at room temperature were plated in a 25-well microtiter plate and irradiated with visible light using a slide projector (Novamat 515 AF Braun), with the lens situated 55 cm below the plate (sitting on a glass support). The output of visible light from the slide projector was measured using a DSE-100X Digital Radiometer and a DIX-555A sensor (Spectronics Corp. Westbury, NY). The light intensity at 520

nm was 1.5 mW/cm^2. After irradiation for 30, 60, and 120 min, the resulting decrease in the absorbance of RNO at 440 nm was measured using a spectrophotometer. Control experiments included nonirradiated mixtures and irradiated mixtures with eosin-containing liposomes lacking TNP-DPPE and liposomes lacking both TNP-DPPE and eosin-lipid.

Binding of TNP-Targeted SUVs to B Lymphoma Cells

A cell pellet of 2×10^6 A20-HL cells was incubated with ^{3}H-DPPC-labeled TNP-DPPE:egg PC:cholesterol SUVs (mole ratio of TNP-DPPE:egg PC:cholesterol is 2:49:49 with trace amounts of ^{3}H-DPPC) in 0.2 ml of PBS (pH 7.4) at 4°C for 60 min. After centrifugation at 1500 rpm (400*g*) and three washes with 5 ml of cold PBS, the cell pellet was resuspended in 0.5 ml of PBS and the amount of cell-bound TNP-bearing liposomes was quantitated from the cpm of the washed pellet suspension after dissolving it in 5 ml of ready value liquid scintillation cocktail (Beckman Instruments, Inc., Fullerton, CA). All assays were performed in triplicate tubes. Control experiments included using the control A20-2J cells, as well as SUVs lacking TNP-DPPE or SUVs containing asialoGM$_2$ instead of TNP-DPPE.

Treatment of B Cell Lines with TNP-Targeted Eosin-Containing Liposomes and Irradiation with Visible Light

To 0.35 ml of B cells ($0.5-1.0 \times 10^6$/ml) in RPMI 1640 without phenol red, 0.35 ml containing varying concentrations of TNP-targeted eosin-containing SUVs was added to the cells in test tubes. After incubation with shaking at 37°C in the dark under 5% CO_2, the cells were centrifuged at 1500 rpm and washed once with 5.0 ml of RPMI 1640. In preliminary experiments the incubation time was varied from 15 to 60 min. The incubation time had little effect, hence 15 min was generally used. In some experiments the cells were incubated at 4°C as indicated in Table 1. The washed cell pellet in each tube was resuspended in 0.7 ml of RPMI 1640 and plated in triplicate in two 96-well flat-bottom microtiter culture plates (Becton–Dickinson, Falcon 3072). One plate was kept as a dark control and the duplicate plate was irradiated with visible light for 40 min as described above. The effect of the treatment on B cell function was determined by measuring B cell uptake of [^{3}H]thymidine.

Uptake of [^{3}H]Thymidine by B Cells

After irradiation with visible light or incubation in the dark, each well (in both the irradiated and nonirradiated plates) was supplemented with 0.1 ml of cell culture medium and 1 μCi of methyl[^{3}H]thymidine (sp act 0.05 Ci/mol, Dupont NEN Products, Boston, MA). After 20 hr incubation at 37°C in the dark under 5% CO_2, the cells were harvested and processed for counting in a beta counter.

Inhibition of Effect of TNP-Targeted Liposomal Eosin on B Cell Lines by Anti-IgM Antibody or TNP-Conjugated KLH

A20-HL cells (7×10^5) were resuspended in 0.05 ml of rat anti-mouse IgM (1/40 dilution in 1% BSA/PBS), mouse anti-rat IgG2a (1/40 dilution in 1% BSA/PBS), or 1% BSA/PBS without antibody in 12-ml polystyrene tubes. After 30 min incubation in the dark at 37°C under 5% CO_2, the cells were washed three times with RPMI 1640. The cell pellets were resuspended in 0.2 ml RPMI 1640 without phenol red and treated with 0.25 ml of TNP-targeted eosin-containing SUVs in PBS (pH 7.4) and light as described above.

A20-HL (0.35 ml) or A20-2J cells (1×10^6 cells/ml) in RPMI 1640 were mixed with 0.05 ml of varying concentrations of TNP_{30}-KLH from 3 to 300 μg/ml KLH in PBS (pH 7.4) in 12-ml polystyrene tubes. After a 15-min incubation at 37°C under 5% CO_2, 0.3 ml of TNP-targeted eosin-containing SUVs in PBS (pH 7.4) or 0.3 ml of PBS only, was added to the tubes and the cells were treated with light as described above.

Treatment of Mixtures of Cells

A20-HL cells (4.7×10^5) and 2.3×10^5 A20-2J cells (in RPMI 1640 without phenol red) were combined in 12-ml polystyrene tubes in a final volume of 0.35 ml. Control tubes containing either 7×10^5 A20-HL or A20-2J cells alone were also prepared. To each tube, 0.35 ml of PBS or TNP-targeted eosin-containing SUVs (mole ratio of TNP-DPPE:eosin-DPPE:egg PC:cholesterol, 1:6:48:45) was added. After 15 min incubation at 37°C in the dark under 5% CO_2, the tubes were washed once with 5 ml RPMI 1640 and the cell pellet was resuspended in 0.7 ml RPMI 1640. The total cell suspension from each tube was transferred to a well in a 25-well flat-bottom culture plate and irradiated. After irradiation, each well was supplemented with 0.7 ml of cell culture medium. After 20 hr incubation at 37°C in the dark under 5% CO_2, each well was prepared for flow microfluorimetric analysis and the number of live A20-HL and A20-2J cells was determined.

Flow Microfluorimetric Analysis of the Mixture of Cells

The cell suspension from each well of the 25-well culture plate was transferred to a 5-ml polystyrene tube and washed three times with washing medium (PBS containing 3% fetal calf serum) and the cell pellet was labeled with biotinylated anti-IgM antibody and streptavidin–phycoerythrin as described earlier. Sample tubes containing A20-HL cells were incubated for 30 min at 4°C in the dark with 0.1 ml of biotinylated rat anti-mouse IgM diluted 1:40 with washing medium.

Sample tubes containing only control A20-2J cells were incubated with 0.1 ml of washing medium alone. After washing three times, the cells labelled with biotinylated antibody were stained for 30 min at 4°C in the dark with 0.1 ml of streptavidin–phycoerythrin conjugate diluted 1/400 with washing medium, while the tubes containing only control A20-2J cells were incubated again with 0.1 ml of washing medium. After washing three more times, 1.0 ml of washing medium and 1.5 μl of 1 mg/ml propidium iodide were added to each of the sample tubes and analyzed by FACScan. For each sample tube, dead cells were excluded by gating out propidium iodide-stained cells and the live population of A20-HL and A20-2J cells was determined. The fluorescence of the A20-HL cell population is due to labeling with anti-IgM, while the fluorescence of the A20-2J population is due to its autofluorescence.

RESULTS

Retention of Eosin-DPPE in TNP-Bearing Eosin-Containing SUVs

The retention of eosin-DPPE in the lipid bilayers of the TNP-bearing liposomes was tested by applying the liposomes to a gel filtration column. The absorbance at 528 nm due to eosin and the PC radioactivity measurements gave similar elution profiles in which two symmetrical peaks were separated (data not shown). One sharp peak was eluted with the void volume of the column, and a second broader peak was distributed in the internal volume of the column and eluted later. Similar behavior has been described previously for SUVs (41). The peak eluting in the void volume is due to a larger more heterogeneous population than that eluting later. The ratio of eosin-DPPE:liposomal phospholipid of the eluate was the same as the initial ratio before the column. The ratio remained the same even after a second application to the column, indicating that the charged eosin-DPPE did not diffuse out of the liposomes and that binding of eosin to DPPE did not confer water solubility to the lipid.

Singlet Oxygen Production by TNP-Targeted Eosin-Containing Liposomes

Fluorescent compounds excited to the triplet state by absorption of light can transfer their energy to TNP rather than to oxygen, giving singlet oxygen (42). Therefore the effect of incorporation of TNP-DPPE into eosin-containing SUVs on their production of singlet oxygen was determined from the decrease in absorbance of RNO at 440 nm (Fig. 1). Unhaptenated eosin-containing liposomes result in a slightly greater decrease in absorption of RNO than those containing TNP-DPPE, indicating that TNP-DPPE had only a small effect in decreasing the amount of singlet oxygen produced from eosin.

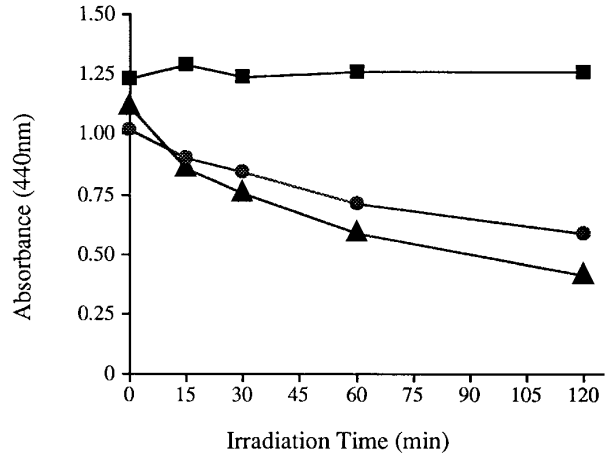

FIG. 1. Dependence of bleaching of RNO in the presence of histidine and liposomal eosin on irradiation time. DMPC SUVs were mixed with 10 m*M* histidine and 30 μM RNO in 0.01 *M* PBS (pH 7.4) and irradiated with visible light. The decrease in the absorbance of RNO at 440 nm with irradiation time indicates the production of singlet oxygen by eosin-containing liposomes. TNP-DPPE eosin SUVs (●), eosin SUVs lacking TNP-DPPE (▲), SUVs lacking both eosin and TNP-DPPE (■).

Expression of Membrane IgM on Target B Cells

To confirm the expression of surface TNP-specific IgM receptors on the A20-HL cells, the cells were incubated with biotinylated anti-IgM followed by the streptavidin–phycoerythrin conjugate and analyzed by flow microfluorimetric analysis. As a negative control, A20-2J B lymphoma cells, which are identical to the A20-HL cells except they lack the genes for the antigen surface receptor, were also labeled. A20-HL cells give a positive fluorescent signal for cell surface IgM whereas the control cells show only background fluorescence (Fig. 2).

Binding of TNP-Targeted Eosin-Containing SUVs to the TNP-Specific B Cells

The A20-HL B cells bind and internalize TNP conjugated to the protein (KLH) via their membrane anti-TNP IgM receptors (43). To determine whether these B cells also bind the TNP specifically when it is presented on the liposomal surface, ^{3}H-labeled TNP-DPPE/egg PC/cholesterol liposomes were incubated with anti-TNP A20-HL and control A20-2J B cells and binding was determined by measuring the cell-associated radioactivity after extensive washing of the cells. Untargeted liposomes lacking TNP-DPPE and liposomes bearing the irrelevant lipid antigen asialoGM_2 were used as negative controls. TNP-targeted liposomes bound to the A20-HL B cells to a greater extent than to the control A20-2J cells (Fig. 3). Furthermore, A20-HL cells bound much less control liposomes (untargeted or targeted with the glycolipid) compared to TNP-targeted liposomes. Control liposomes bound to

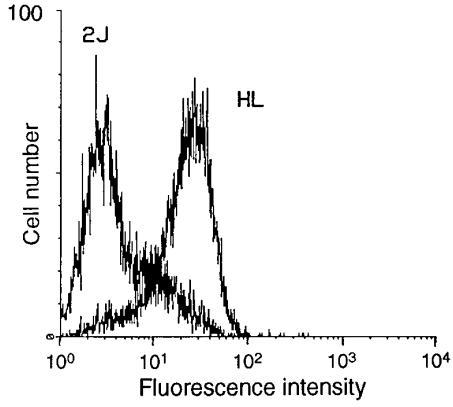

FIG. 2. Expression of surface IgM on B lymphoma cells measured by flow microfluorimetric analysis. TNP-specific B cells (A20-HL) were stained with biotinylated anti-mouse IgM followed by a streptavidin–phycoerythrin conjugate. Approximately 10^4 cells were analyzed and plotted as a function of fluorescence intensity. Background fluorescence due to nonspecific binding of the streptavidin–phycoerythrin conjugate has been subtracted from each tracing. As a control experiment, the control B cells (A20-2J) which do not express surface IgM were also analyzed.

both the target B cells and the control B cells to the same extent.

TNP-Targeted Eosin-Containing SUVs Affect B Cell Proliferation

TNP-specific B cells (A20-HL) and control B cells (A20-2J) were incubated for 15 min at 37°C with TNP-targeted eosin-containing egg PC/cholesterol SUVs and irradiated with visible light as described under Materials and Methods. Incubation for a longer time, 60 min, did not affect the results. After irradiation, cell proliferation was determined from uptake of [^{3}H]thymidine during a 20-hr incubation period. The effect of light irradiation alone on the cells was usually negligible. Furthermore, the proliferation of liposome-treated cells which were not irradiated was not inhibited. Because of the effect of TNP-DPPE in partially inhibiting production of singlet oxygen (Fig. 1), the effect of untargeted liposomal eosin lacking TNP-DPPE is not an appropriate control for specificity and was not determined. Instead, the effect of TNP-targeted liposomal eosin on the control A20-2J cells was compared to that on the anti-TNP A20-HL cells in order to determine specificity. In preliminary experiments the two liposome fractions obtained by gel filtration chromatography were tested separately. However, they had similar effects on the cells to each other and to the unfractionated mixture. Therefore the unfractionated liposomes were used in subsequent experiments.

The results from three typical experiments testing different preparations of liposomes are shown in Fig 4. The concentration of liposomal eosin required to inhibit the cell proliferation by 50% (ED_{50}) was significantly lower for TNP-specific B cells than for control B cells (Fig. 4). The occurrence of specific inhibition was highly reproducible, but the degree of specificity and the ED_{50} values varied from one experiment to another. The ED_{50} for anti-TNP B cells ranged from 10^{-7} to 10^{-5} *M* eosin, depending on the liposomal preparation and the eosin-DPPE preparation. Out of 44 experiments using different liposomal preparations, 37 resulted in a specific targeting effect in which the ratio of ED_{50} values for the control cells relative to the anti-TNP cells was 5 or greater.

When the A20-HL cells were incubated with TNP-conjugated KLH before treatment with the TNP-targeted phototoxic liposomes, the inhibition of cell proliferation by the targeted phototoxic liposomes was almost completely abrogated especially at high KLH-TNP to liposomal TNP ratios. Even at a KLH-TNP to liposomal TNP ratio of 0.02, inhibition was significantly decreased (Fig. 5). The smaller degree of inhibition of proliferation of the control A20-2J cells was not affected by the same treatment. When anti-mouse IgM antibody was added to the A20-HL cells before treatment with TNP-targeted phototoxic liposomes, the inhibition of cell proliferation by the haptenated phototoxic liposomes was also significantly reduced (Fig. 6). Pretreatment with anti-rat IgG, which does not bind to the IgM B cell receptor, did not inhibit the antigen-specific effect. These results indicate that the TNP-targeted liposomal eosin inhibits anti-TNP B cell proliferation by binding to the anti-TNP IgM receptor.

Incubation of the cells with liposomal eosin at a lower temperature (4°C), in order to inhibit endocytosis, did not affect the concentration of eosin required to inhibit

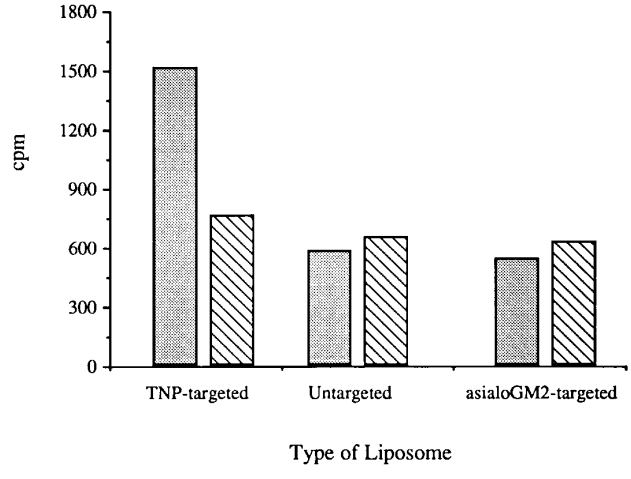

FIG. 3. Binding of radioactive liposomes to TNP-specific B cells (A20-HL) (▨) and control B cells (A20-2J) (▩). Similar numbers of A20-HL or A20-2J cells were incubated with untargeted and antigen-targeted ^{3}H-liposomes for 60 min at 4°C. The amount of cell-bound liposomes was determined from the radioactivity of the cells after they had been washed three times with 5 ml of PBS. The binding between TNP-specific B cells and TNP-targeted liposomes is antigen-specific. The data represent mean values of triplicate determinations. Standard deviations were less than 5% of mean values.

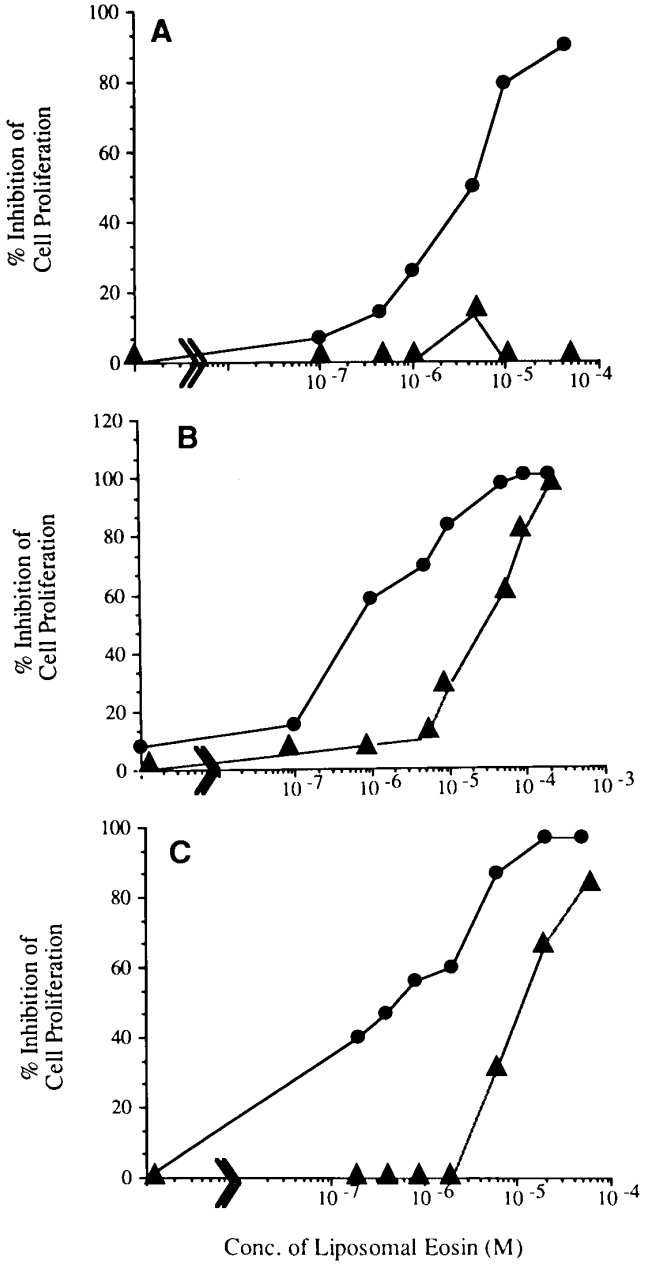

FIG. 4. TNP-targeted eosin-containing liposomes inhibit the proliferation of B lymphoma cells. Varying concentrations of TNP-targeted liposomal eosin were incubated with TNP-specific B cells (●) and control B cells (▲) and irradiated as described under Materials and Methods. After 20 hr at 37°C, [^{3}H]-thymidine uptake was determined. The data points represent the mean value of triplicate determinations and are plotted as a percentage relative to the corresponding value from similarly treated wells maintained in the dark. Standard deviations were always less than 10% of the values indicated and usually less than 5%. Three typical experiments are shown in A, B, and C. The data points on the *Y* axis represent the values in the absence of liposomal eosin.

proliferation (experiment 1, Table 1), indicating that the liposomal eosin probably acted on the plasma membrane and did not need to be internalized to exert its effect. The possibility that nonspecific binding might

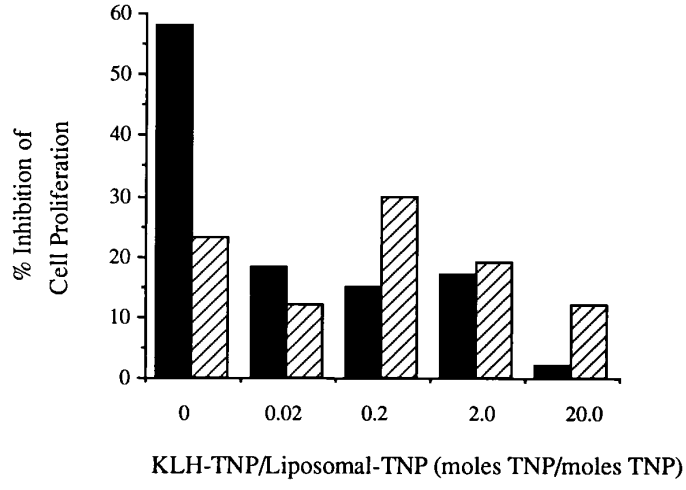

FIG. 5. Blocking of specific targeting effect on TNP-specific B cells by TNP-conjugated KLH. TNP-specific B cells (■) were pretreated with various concentrations of TNP_{30}-KLH at 37°C. After 15 min, TNP-targeted liposomal eosin (10^{-5} *M* eosin) was added and the cells were incubated for another 15 min at 37°C, washed, and irradiated. After 20 hr, the cell proliferation was determined by measuring the uptake of [^{3}H]-thymidine by the treated cells. The data represent mean values of triplicate determinations and are plotted as a percentage relative to the corresponding value from similarly treated wells maintained in the dark. In a control experiment, control B cells (A20-2J) which do not bind the TNP-KLH specifically via surface IgM receptors were also tested (▨). Mole ratio of TNP from TNP_{30}-KLH to that from TNP-DPPE in liposomes is indicated.

be reduced by incubation at 4°C rather than at 37°C was also considered. Cells were incubated at 4°C and washed in order to remove any liposomes bound non-

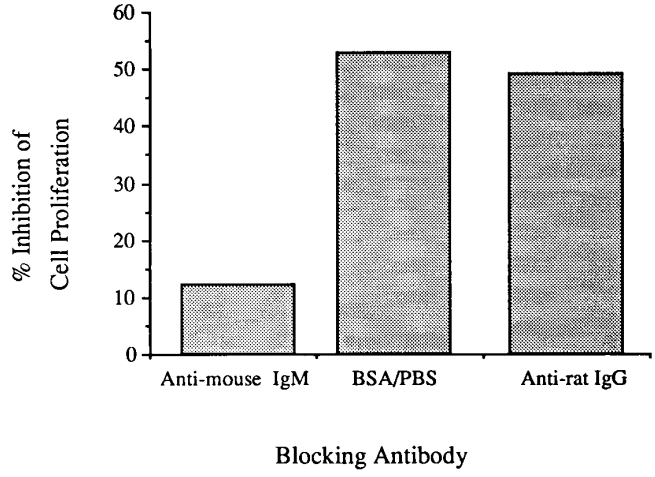

FIG. 6. Blocking of the specific targeting effect on TNP-specific B cells by anti-IgM antibody. The data were obtained as described in the legend to Fig. 5 except A20-HL cells were preincubated with anti-mouse IgM in 1% BSA/PBS or with an anti-rat IgG antibody that does not bind to the target B cells, or were incubated with 1% BSA/PBS only. After 30 min, the cells were extensively washed and treated with TNP-targeted liposomal eosin. Standard deviations were less than 4% of the values shown.

TABLE 1

Effect of Incubation Temperature of B Cells with Liposomal Eosin

Expt.	Incubation procedure[a]	ED_{50} (*M*) A20-2J	ED_{50} (*M*) A20-HL	Specificity[b]
1	37°C 60 min	5×10^{-5}	3.5×10^{-6}	14.3
	4°C 60 min	3×10^{-5}	3×10^{-6}	10
2	37°C 15 min, wash	$>6.5 \times 10^{-5}$[c]	1.5×10^{-5}	>4.3
	4°C 15 min, wash, 37°C 15 min	$>6.5 \times 10^{-5}$[c]	8.2×10^{-6}	>7.9
3	4°C 20 min, 37°C 20 min	4.5×10^{-5}	2.5×10^{-6}	18
	4°C 20 min, wash, 37°C 20 min	4.5×10^{-5}	2.5×10^{-6}	18

[a] B Cells were incubated with liposomal eosin at temperatures shown and plated in microtiter plates. After plating, cells were irradiated with visible light for 40 min. ED_{50} values were determined from curves similar to those in Fig. 4.

[b] Ratio of ED_{50} for liposomal eosin required to inhibit control A20-2J cells relative to that for anti-TNP A20-HL cells. A higher value indicates greater specificity.

[c] The highest concentration used in this experiment.

specifically with low affinity, before incubation at 37°C. However, the degree of specificity was similar to that on cells incubated only at 37°C (experiment 2) or not washed in between a 4°C incubation and a 37°C incubation (experiment 3, Table 1).

Use of auxiliary phospholipids with a higher phase transition temperature, DMPC, DPPC, DSPC, and SM (all with cholesterol), decreased the specificity of the effect relative to that observed with egg PC/cholesterol (Table 2). The concentration of DMPC and DPPC liposomal eosin required to inhibit proliferation of anti-TNP A20-HL cells was similar to that for egg PC/cholesterol liposomal eosin as indicated by the ED_{50} values in Table 2, but a much lower concentration was able to inhibit the control A20-2J cells compared to egg PC/cholesterol liposomal eosin. For DSPC less liposomal eosin was required to inhibit both cell lines. In all three cases, DMPC,- DPPC-, and DSPC-containing liposomes had a much greater non-specific effect on the control A20-2J cells. SM/chol liposomes, however, were less effective on both cell lines.

TABLE 2

Dependence of ED_{50} and Specificity of Liposomal Eosin on Lipid Composition of Liposomes Compared to Egg PC/cholesterol[a]

	ED_{50}(M)[b] A20-2J	ED_{50}(M)[b] A20-HL	Specificity[c]
DSPC/chol	3.0×10^{-6}	1.5×10^{-6}	2.0
Egg PC/chol	6.5×10^{-5}	9.0×10^{-6}	7.2
DPPC/chol	6.5×10^{-7}	2.5×10^{-7}	2.6
Egg PC/chol	1.6×10^{-6}	2.6×10^{-7}	6.2
DMPC/chol	1.1×10^{-6}	4.0×10^{-7}	2.8
Egg PC/chol	2.0×10^{-5}	6.0×10^{-7}	33.0
SM/chol	1.5×10^{-6}	5.0×10^{-7}	3.0
Egg PC/chol	7.0×10^{-7}	1.4×10^{-7}	5.0

[a] Liposomal eosin was tested on anti-TNP A20-HL and control A20-2J cells.

[b] ED_{50} value for liposome composition indicated compared to that of egg PC/chol liposomes which were tested at the same time. Each pair was tested in a different experiment. ED_{50} values were determined from curves such as those shown in Fig. 4. All liposomes contained 2 mol% TNP-DPPE and 5 mol% eosin-DPPE in addition to the lipids listed.

[c] Ratio of ED_{50} for liposomal eosin required to inhibit control A20-2J cells relative to that for anti-TNP A20-HL cells. A higher value indicates greater specificity.

Selective Killing of Anti-TNP-B Cells by TNP-Targeted Eosin-Containing SUVs in a Cell Mixture

The TNP-targeted phototoxic liposomes were added to a mixed cell population of the two B lymphoma cell lines, A20-HL and A20-2J, to test the selectivity of the treatment when antigen-specific and control cells were mixed together. The cytotoxic effect on each cell line was determined by FACS analysis. The live population of the total cells (A20-HL and A20-2J) was analyzed before and after treatment by excluding the dead cells with propidium iodide dye. In the live cell mixture, the target anti-TNP B cells were distinguished from the control B cells by labeling the mixture with biotinylated anti-IgM antibody which binds only to the surface IgM receptors on the target B cells (Fig. 2). After treatment, the live A20-HL B cell population was significantly reduced compared to the control B cell population which was not affected in the mixture (Fig. 7 and Table 3). The specificity of the liposomal eosin for the anti-TNP A20-HL cells was even greater when the cells were mixed together than when they were treated individually (Table 3).

DISCUSSION

Selective targeting of haptenated liposomal methotrexate to hapten-reactive B cells was attempted previously using the B myeloma cell line MOPC 315 as the target B cell (30, 49). Although the MOPC 315 cells did bind the dinitrophenol (DNP)-targeted liposomes specifically via

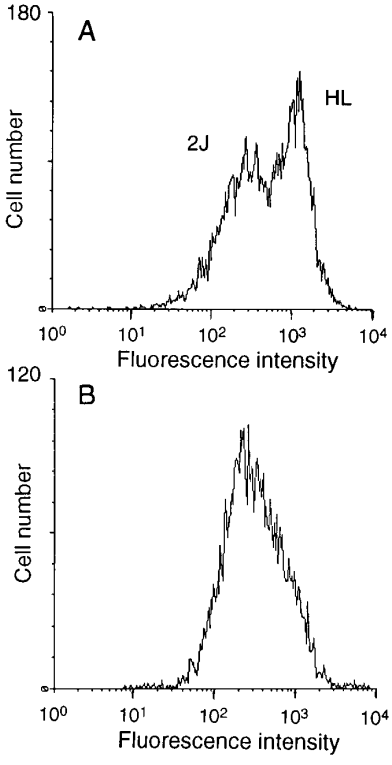

FIG. 7. Effect of TNP-targeted phototoxic liposomes on the viability of B lymphoma cells in a mixture of TNP-specific (HL) and control B cells (2J) determined by flow microfluorimetric analysis. (A) Cell mixture in the absence of TNP-targeted liposomal eosin. (B) Cell mixture treated with TNP-targeted liposomal eosin (2×10^{-4} *M* eosin). As indicated in Table 3 most of the anti-TNP HL cells have been selectively eliminated. A20-HL (HL) and A20-2J (2J) B lymphoma cells (to give a total of 7×10^5 cells) were mixed in 12-ml test tubes and incubated with TNP-targeted eosin-containing liposomes at 37°C. After 15 min, the cells in each tube were washed, plated, and irradiated. After 20 hr in the dark, the cells in each well were stained with biotinylated anti-IgM antibody and streptavidin-phycoerythrin conjugate for analysis by flow microfluorimetry. Just before analysis, propidium iodide was added to each sample tube. Dead cells were excluded by gating out propidium iodide-stained cells and the live populations of HL and 2J cells were counted and the percentage of total cells was determined. 2J cells were counted over a range of fluorescence intensity of 30–550. HL cells were counted over the range 500–10,000. Control tubes containing TNP-specific B cells, control B cells, or untreated cell mixtures were also prepared.

surface DNP-specific receptors, antigen-specific uptake of methotrexate was not achieved because the liposomes were not internalized by the target B cells. In addition, the expression of the antigen-specific receptors on MOPC 315 cells may have been too low to obtain an antigen-specific effect. We found by FACS analysis that only a fraction of the MOPC 315 cell population expresses the receptors (data not shown) and others have reported this cell line expresses less surface Ig compared to other B myeloma cell lines (44).

In this study we made use of the TNP-specific B lymphoma cell line A20-HL produced by transfection of the B lymphoma cell line A20-2J with the genes for the membrane form of IgM specific for TNP (33). The expression of the IgM receptor on the A20-HL cells but not on the control A20-2J cells was confirmed by FACS analysis. Therefore, the A20-HL B cell line is an appropriate model B cell to study specific antigen receptor-mediated targeting and killing by antigen-targeted liposomal drugs. Liposomes bearing TNP on their surface were used for the specific delivery of the photosensitizer eosin to the target B cells. Photosensitizers offer several advantages over encapsulated cytotoxic compounds as liposomal drugs (45). Their cytotoxicity is easily controlled since they are activated only upon exposure to light and their toxic byproducts are active for only a short time. This increases their potential for *in vivo* application since the phototoxic liposomes sequestered by the reticuloendothelial system can be shielded from irradiation to prevent unwanted side effects. Photosensitizers are cytotoxic without the need to be internalized by cells because their activation by light generates toxic singlet oxygen molecules which can modify cell membrane components to result in cell damage or death. In addition, the nonspecific effects of targeted photosensitizers are minimized since the action of the singlet oxygen molecules is confined to cells in the immediate vicinity of the liposomes at the time of irradiation due to the short half-life and diffusion path of these toxic species.

Eosin was chosen as the photosensitizer because it could be coupled to a phospholipid for easy binding to liposomes and because it did not transfer energy readily to TNP. Cells with eosin-labeled cell surface proteins have been lysed after irradiation with visible light (35, 46). To reduce loss from the liposome, eosin was coupled to a phospholipid and incorporated into the lipid bilayers of the liposomes. After incorporation into the liposomes, eosin retains its ability to generate the production of singlet oxygen molecules, although reduced somewhat in the presence of TNP. This reduction may be due to the transfer of energy from the excited triplet state of eosin to TNP rather than to oxygen to produce singlet oxygen. Kimura *et al.* (42) showed a drastic decrease in liposomal pyrene's fluorescence intensity in the presence of TNP. We found the ability of liposomal pyrene to produce singlet oxygen to be inhibited in the presence of TNP even more than that of eosin (unpublished).

Binding between the A20-HL cells and the TNP-targeted SUVs was observed and found to be antigen-specific. The TNP-targeted eosin-containing liposomes inhibited the cell proliferation of the TNP-specific B cells significantly more than that of the control B cells. Under conditions which did not favor internalization or receptor-mediated endocytosis by the target B cells (4°C), the phototoxicity of the TNP-targeted liposomal eosin was not affected supporting the conclusion that eosin does not require internalization to be cytotoxic. The antigen-specific targeting effect by the TNP-targeted phototoxic liposomes was highly reproducible, but the degree of specificity as indicated by the ratio

TABLE 3

Specificity of TNP-Liposomal Eosin on A20-HL and A20-2J Cells Individually and When Mixed Together Determined by FACS Analysis[a]

Eosin (M)	Total cells		A20-HL			A20-2J		
	% cells[b]	% inhibition[c]	% live cells[d]	% cells[b]	% inhibition[c]	% live cells[d]	% cells[b]	% inhibition[c]
				Cells treated individually				
0				76			88	
1×10^{-4}				50.5	34		72.1	18
2×10^{-4}				32.8	57		67	24
				Cells Treated Mixed Together				
0	86		55.6	47.8		45.6	39.2	
1×10^{-4}	60	30	31.9	19.1	60	70.5	42.3	0
2×10^{-4}	53	38	31.5	16.7	65	71	37.6	4.1

[a] 7×10^5 anti-TNP A20-HL and control A20-2J B cells were treated individually or mixed together (to give a total of 7×10^5 cells) in test tubes and treated with TNP-targeted eosin-DPPE containing liposomes at 37°C for 15 min. The cells were washed, plated in 25-well culture plates, irradiated with light, and incubated at 37°C for 20 hr in the dark. The A20-HL cells in each well were stained with biotinylated anti-IgM antibody and steptavidin-phycoerythrin conjugate. Dead cells were excluded by gating out propidium iodide-stained cells. A20-2J cells were detected and quantitated from natural autofluorescence. Data from one of three similar experiments are shown.

[b] Percentage of total cells which are alive.

[c] Percentage inhibition relative to number of live cells in the absence of eosin.

[d] Percentage of total live cells due to each cell line.

of ED_{50} values for anti-TNP and control B cells between treatment experiments was variable. This may have been due to uncontrolled differences in the liposome preparations used for different experiments and/or to the possible shedding of the antigen-specific receptors from the surface of the target B cells to variable extents. This specific toxic effect could be inhibited by TNP_{30}-KLH and anti-IgM antibody, indicating that it is mediated via the antigen receptor. When the target B cells were mixed with the control B cells and treated with TNP-targeted liposomal eosin, there was a significant reduction in the number of live target B cells but the control B cell population was not affected, indicating low nonspecific damage to bystander cells. At higher concentrations liposomal eosin also inhibits the control B cells. This is probably a result of the nonspecific adsorption of the liposomes to the control B cells as indicated in the binding studies.

TNP_{30}-KLH was effective at inhibiting the effect of the TNP-targeted liposomal eosin at much lower TNP concentrations than present in the liposomes, indicating that the KLH-bound TNP binds to the surface IgM receptor with a higher affinity than the liposomal TNP. The TNP on the surface of the egg PC/chol liposomes may not be highly exposed for binding to the Ig receptor. Use of liposomes containing the higher phase transition lipids DPPC, DSPC, or SM was expected to improve the targeting ability of TNP-DPPE and specificity of the liposomal eosin, since these lipids have been found to cause greater binding of anti-DNP and TNP antibody or B cells to liposomes containing DNP- or TNP-DPPE (32, 47, 31). Liposomes containing these lipids also induce a stronger humoral response to haptenated lipids (48). This effect is probably due to decreased penetration of the hapten and the hydrophobic caproyl spacer group connecting the hapten to ethanolamine into the bilayer or the apolar/polar interface region of the less fluid bilayers compared to egg PC/cholesterol (42). However, use of these lipids increased the effect on the control cells, suggesting that they increased nonspecific binding of the liposomes to the cells or increased transfer of the DPPE-eosin to the cells during transient nonspecific contact of the liposomes with the B cells due to a lower solubility in the less fluid lipids.

Other more polar lipid haptens such as the carbohydrates of glycolipids are expected to be exposed more in the more fluid egg PC/chol and DMPC/chol liposomes since these bilayers are thinner than DPPC/chol and DSPC/chol with their longer fatty acid chains. Indeed this has been found to be the case for galactosylceramide and cerebroside sulfate (26, 27). Thus for targeting of phototoxic drugs to anti-glycolipid B cells, use of egg PC/chol or DMPC/chol liposomes should maximize recognition by and binding to antigen-specific B cells and minimize nonspecific effects on other cells.

In summary, specific elimination of the model anti-TNP B cell line A20-HL using TNP-targeted phototoxic liposomes has been demonstrated *in vitro*. Glycolipids and other disease-associated lipid antigens should be able to target phototoxic liposomes to specific B cell clones with the appropriate surface receptors.

ACKNOWLEDGMENTS

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Note added in proof. While this paper was in press we became aware of a recent study of J-C. Grivel, K. Crook, and L. Leserman (*Immunomethods* 4, 223, 1994) which demonstrated protein antigen-specific targeting of methotrexate by liposomes bearing hen egg lysozyme bound to the surface to transgenic B lymphoma cells transfected with the DNA for surface Ig specific for this protein. These B cells were able to endocytose the liposomes and thus internalize their encapsulated methotrexate.

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