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Uptake of antibody directed cytotoxic liposomes by CD3 on human T cells

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SUMMARY

Using polyclonal human T cells and anti-CD3 monoclonal antibodies we have shown that small unilamellar liposomes covalently coupled with protein A become bound to T cells and not to B cells and that the binding was a specific liposome–antibody-receptor interaction. Intracellular delivery of liposome contents was demonstrated by the use of encapsulated carboxyfluorescein and flow cytometry and the transfer of membrane-bound liposomal carboxyfluorescein was virtually complete in 30 min. Liposomes containing methotrexate inhibited the growth of PHA-stimulated peripheral blood lymphocytes by 90%, after 48 h incubation. Potential applications are proposed in the study of the behaviour of surface membrane components and in T cell depletion and purging of bone marrow.

Keywords cytotoxic liposomes cells bone marrow transplantation

INTRODUCTION

Liposomes can be targeted to specific cells using antibodies to cell surface antigens following which they may or may not be endocytosed (Machy, Barbet & Leserman, 1982). Previous studies have shown the uptake of antibody-directed liposomes by T and B lymphoid cell lines using MHC molecules (Machy *et al.*, 1982; Huang, Kennel & Huang, 1983) but in order to contemplate clinical usage targeting must be restricted to antigens which are limited to specific cell types. In the context of allogeneic bone marrow transplantation, targeting of cytotoxic lymphocytes to T cells might be of value as a method of T cell depletion of marrow which effectively reduces the frequency and severity of graft-versus-host disease (Prentice *et al.*, 1984). We have therefore investigated whether liposomes can be targeted specifically to the CD3-T_i complex on T cells and whether the liposome contents are internalized.

The targeting system which has been used involves the covalent attachment of protein A to the liposome membrane followed by incubation with lymphocytes which have been coated with anti-CD3 antibody in a previous step. Using this method we have studied the effectiveness of liposome-encapsulated methotrexate (MTX) in inhibiting lymphocyte growth in culture.

MATERIALS AND METHODS

Liposome synthesis

Small unilamellar vesicles or liposomes (SUV) were synthesized and covalently coupled to protein A (PA) after the method of Leserman *et al.* (1980). Purified carboxyfluorescein (CF), 40 mM or 200 mM, or 20 mM MTX (Bristol Myers) was encapsulated. Approximately 20% of the available protein became coupled to liposomes as measured by radioactive counting (SUV-PA). The final concentration of liposomes was 2·5 mM (total lipid) and that of protein A 50 μg/ml.

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Antibodies

UCHT1 is a monoclonal anti-CD3 antibody raised in mice. The original antibody is subclass IgG_1 but IgG_{2b} and IgG_{2a} switch-variants of the same antibody have also been produced (Beverley & Callard, 1981). The IgG_{2b} and IgG_{2a} subclasses are able to bind protein A at pH 7·4 whereas the IgG_1 subclass is not. Except where specifically mentioned, the IgG_{2b} switch-variant has been used in the experiments. Saturating concentrations (> 5 μg/ml) of culture supernatant and ascitic fluid were used. Fluorescent goat anti-mouse immunoglobulin (GαM-FITC) was obtained from Nordic Laboratories.

Cell preparation

Fresh heparinized peripheral blood from healthy volunteers was diluted with cell culture medium (RPMI 1640, Gibco) and the mononuclear cells separated on a Ficoll-Hypaque gradient (Pharmacia). To remove monocytes, 5 ml of mononuclear cells at 3×10^6/ml in RPMI 1640 and 20% fetal calf serum was incubated overnight at 37°C in a plastic tissue culture flask. Non-adherent cells were removed by gently washing with RPMI 1640. PHA-lymphoblasts were obtained by incubation of peripheral blood mononuclear cells (1×10^6/ml) for 72 h in cell culture medium containing 10% fetal calf serum and purified phytohaemagglutinin (4 μg/ml, Wellcome).

Liposome uptake by peripheral blood lymphocytes
Samples of 2×10^5 cells were stained with monoclonal antibody in microtitre plates as described by Linch *et al.* (1982). Fifty microlitres of SUV-PA or 50 μl of GαM-FITC was added for 30 min at 4°C after which cells were washed three times in ice-cold medium. Flow cytometric analysis was performed on a FACS IV (Becton Dickinson). The effect of temperature on cell fluorescence was studied by placing cells labelled at 4°C with SUV-PA or GαM-FITC in a waterbath at 37°C. At specified intervals, cells were removed and the fluorescence re-analysed on the FACS.

UV microscopy
Cells labelled with fluorescent liposomes or fluorescent second layer antibody were placed on glass slides and sealed under coverslips at 4°C. They were examined immediately under a UV microscope using phase optics and a ×60 magnification oil immersion lens. Modulation of surface fluorescence was studied by allowing the chilled slides to warm on the microscope stage. The temperature of the slide was 25°C after 5 min and 35°C after 20 min. Photographs were taken at 10 min intervals using a standard exposure time of 15 s. The UV source was occluded between examinations to prevent bleaching. All prints were handled identically.

Treatment of cells with ZZAP
PBL were labelled at 4°C with antibody and SUV-PA containing 40 mM CF. After thorough washing in ice-cold medium, fluorescence was measured by flow cytometry at this time and after 60 min at either 4°C or 37°C. Cells were then treated for 15 min at room temperature with medium containing 0·1 M dithiothreitol and 0·1% cysteine-activated papain (NBTS, Edgware) in a modified application of the ZZAP method (Branch & Petz, 1982). After further washing at 4°C cells were re-analysed on the FACS.

Effect of liposome-encapsulated MTX on PHA-lymphoblasts
The proliferation of PHA-lymphoblasts was assessed by measuring the uptake of tritiated deoxyuridine (^{3}H dUr). Two hundred thousand cells per well were plated in triplicate cultures in 96-well microtitre plates. Fifty microlitres of antibody and 50 μl of liposomes were added followed by 100 μl of the original culture supernatant containing 10% FCS and PHA. Cells were cultured in a fully humidified atmosphere of 5% CO_2 at 37°C. One microcurie per well of ^{3}H dUr (Amersham) was added for the last 6 h of culture. Cells were harvested at 24 and 48 h on to fibreglass filters (Whatman) using an Automash (Dynatech). Counts were measured on a scintillation counter set for ^{3}H.

RESULTS

Targeting of protein A bearing liposomes at 4°C
The ability of protein A bearing liposomes to attach specifically to T cells was assessed using peripheral blood lymphocytes (PBL) which are 65–70% T cells, with B cells and non B, non T lymphocytes comprising the majority of the remainder. Because B cells do not express CD3 and hence do not bind UCHT1, they are not detected by fluorescent second layer antibody and do not attach liposomes. The non-T cell population acts as a useful internal control for any non-specific attachment of liposomes.

Table 1. Targeting of protein A bearing liposomes

	% of positive cells	Relative fluorescence intensity
(a) Cells + GαM-FITC	7	27
Cells + SUV-PA	8	29
Cells + UCHT1 + GαM-FITC	68	107
Cells + UCHT1 + SUV-PA	67	104
(b) Cells + UCHT1 + (IgG1) + GαM-FITC	67	96
Cells + UCHT1 (IgG1) + SUV-PA	10	43
Cells + UCHT1 (IgG2b) + GαM-FITC	65	95
Cells + UCHT1 (IgG2b) + SUV-PA	65	91
Cells + UCHT1 (IgG2a) + GαM-FITC	64	97
Cells + UCHT1 (IgG2a) + SUV-PA	65	95

(a) Peripheral blood lymphocytes were incubated with anti-CD3 antibody (UCHT1) and FITC-conjugated goat anti-mouse antibody (GαM-FITC) or protein A bearing liposomes containing 40 mM carboxyfluorescein (SUV-PA). Negative controls were cells incubated with the second layer alone. Fluorescence was measured by flow cytometry in a FACS IV (Becton Dickinson) and the results expressed as the percentage of fluorescence positive cells and the relative fluorescence intensity.

(b) Peripheral blood lymphocytes were incubated with IgG1, IgG2b and IgG2a switch variants of the anti-CD3 antibody UHT1. The ability of these antibodies to bind GαM-FITC and SUV-PA was compared.

Flow cytometric analysis of PBL labelled with UCHT1 antibody and protein A bearing liposomes (SUV-PA) containing 40 mM CF at 4°C is shown in Table 1a. The results are given as the number of fluorescence positive cells and the relative fluorescence intensity (peak channel number). The negative control in each case is cells incubated with the fluorescent layer alone, i.e. without UCHT1, and the positive control is cells incubated with UCHT1 and conventional second layer antibody labelled with FITC (GαM-FITC).

Protein A bearing liposomes attach to approximately equal numbers of PBL (65–70%) as GαM-FITC when UCHT1 is present. When UCHT1 is omitted the cells become negative and the small number of positives is again approximately equal in both cases.

Protein A liposomes therefore bind to cells labelled with antibody. The 30% or so of PBL which are not fluorescent are the B cells and non-B, non-T lymphocytes which, though ontologically closely related to the T cells, do not bear the targeting antibody. To exclude the possibility that T cell binding was a non-specific interaction between cell-bound antibody and liposomes, we compared the IgG_1 and IgG_{2b} switch variants of the antibody. Although the IgG_1 subclass of UCHT1 is present on the cell surface as indicated by its ability to bind GαM-FITC (Table 1(b)), it is unable to bind protein A liposomes. However the IgG_{2b} and IgG_{2a} variants are able to bind liposomes and GαM-FITC equally well and to bind the latter as effectively as the IgG_1. These data demonstrate that the interaction of protein A liposomes with T cells is solely by means of protein A-antibody bridging.

Incubation of cells with protein A liposomes at 37°C
PBL labelled with liposomes or GαM-FITC were incubated at 37°C in order to promote internalization. This initiated a rapid

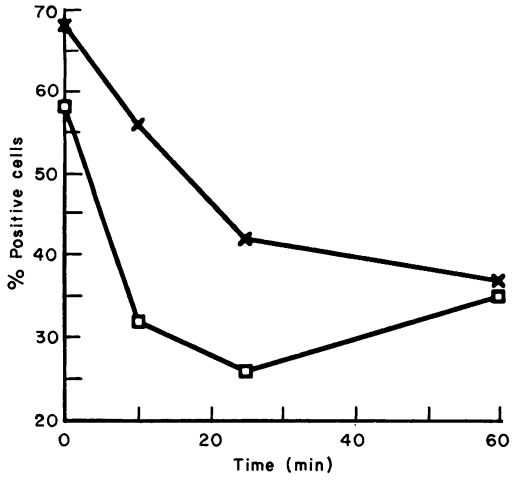


Fig. 1. Modulation of lymphocyte fluorescence at 37°C using 40 mM CF liposomes. Peripheral blood lymphocytes were labelled with anti-CD3 antibody and FITC conjugated goat anti-mouse antibody or protein A liposomes containing 40 mM carboxyfluorescein. Unbound antibody and liposomes were washed off and the cells incubated at 37°C. The percentage of fluorescence positive cells at various time intervals was measured by flow cytometry. The graph is representative of two experiments. (×) FITC-Ab, (□) SUV-PA.

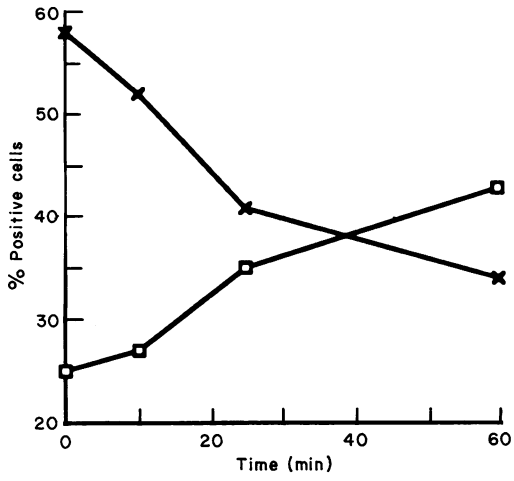


Fig. 2. Modulation of lymphocyte fluorescence at 37°C using 200 mM CF liposomes. Peripheral blood lymphocytes were labelled with anti-CD3 antibody and FITC conjugated goat anti-mouse antibody or protein A liposomes containing 200 mM carboxyfluorescein. Conditions, measurements and symbols were as Fig. 1.

decline in the number of positive cells. Using the FITC label, approximately 50% of cells which were originally positive remained so after 60 min (Fig. 1). Liposome-labelled cells showed a similar rapid fall reaching its nadir of 50% after 30 min. This could be explained by internalization of fluorophore with subsequent dilution in the cytoplasm and weakening of the fluorescent signal or alternatively, shedding of the complexes into the extracellular medium where they would be undetected by the FACS. The experiments were therefore repeated using liposomes containing much higher concentrations of CF, thereby increasing the likelihood of intracellular fluorescence being detected by the FACS. Cells were labelled with liposomes containing 200 mM CF and incubated at 37°C. The opposite effect was now seen (Fig. 2). The number of positive cells was 25% at the start and increased to 46% at 60 min.

Table 2. Effect of carboxyfluorescein concentration on liposomal fluorescence

Concentration of carboxyfluorescein in liposomes	Fluorescence units: Before adding Triton-X	Fluorescence units: After adding Triton-X	Percentage quenching
40 mM	114	454	74·9
200 mM	35	2104	98·4

The fluorescence of two preparations of liposomes containing different concentration of carboxyfluorescein were compared. Intact liposomes were lysed by addition of Triton-X to release the fluorophore and abolish quenching. The percentage quenching =

$$100 - \frac{\text{(Fluorescence before Triton-X)}}{\text{(Fluorescence after Triton-X)}} \times 100$$

Table 3. Removal of membrane-bound liposomes by enzyme

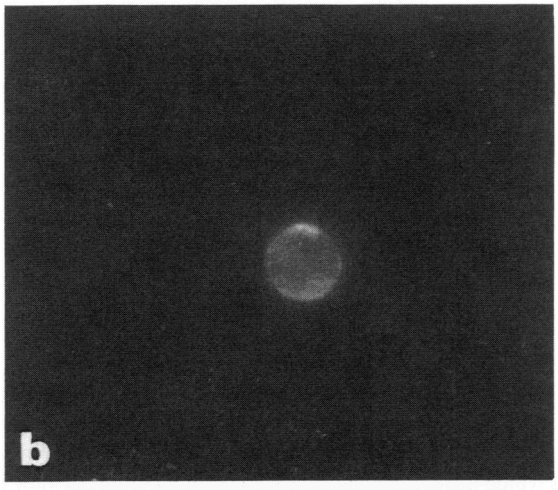
Cells	Incubation	% positive cells	Relative fluorescence intensity
(a) +Ab+SUV-PA	None	72	92
+SUV-PA	None	4	32
(b) +Ab+SUV-PA	60 min at 4°C	72	93
	after enzyme	6	33
(c) +Ab+SUV-PA	60 min at 37°C	33	72
	after enzyme	25	58

Peripheral blood lymphocytes coated with anti-CD3 antibody and protein A bearing liposomes containing 40 mM CF were analysed by flow cytometry and compared with control cells not coated with antibody (a). Cells labelled with antibody and liposome were reanalysed after 60 minutes incubation (b) at 4°C or at (c) 37°C. These were treated with activated papain and dithiothreitol for 15 min at room temperature to remove membrane-bound liposomes and fluorescence was again measured.

The difference in behaviour is explained by the degree of self-quenching in each of these species of liposome. CF liposomes, 200 mM, were considerably less fluorescent than those containing 40 mM CF although they contained approximately five times as much fluorophore (Table 2). Fluorescence was fully released after lysis with Triton-X and the percentage quenching was calculated using the formula:

$$\% \text{ quenching} = 100 - \frac{\text{Fluorescence before Triton-X}}{\text{Fluorescence after Triton-X}} \times 100$$

Thus 40 mM CF liposomes were relatively unquenched (74·9%) compared to those containing 200 mM CF which were



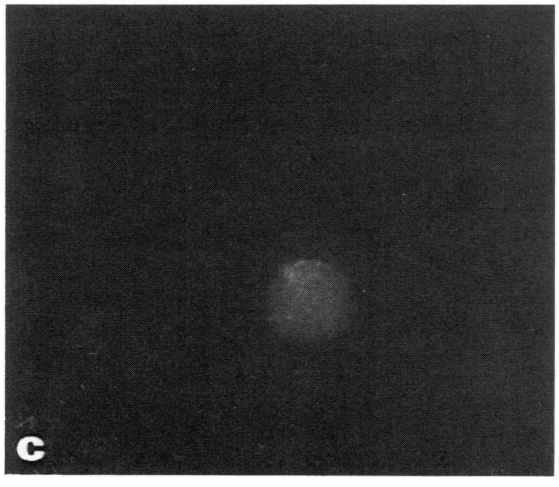


Fig. 3. Fluorescent micrographs of a single peripheral blood T cell labelled with UCHT1 and protein A liposomes containing 200 mM carboxyfluorescein. Photographs were taken at (a) time zero, and (b) 10 min and (c) 20 min after warming from 4°C. See text for details.

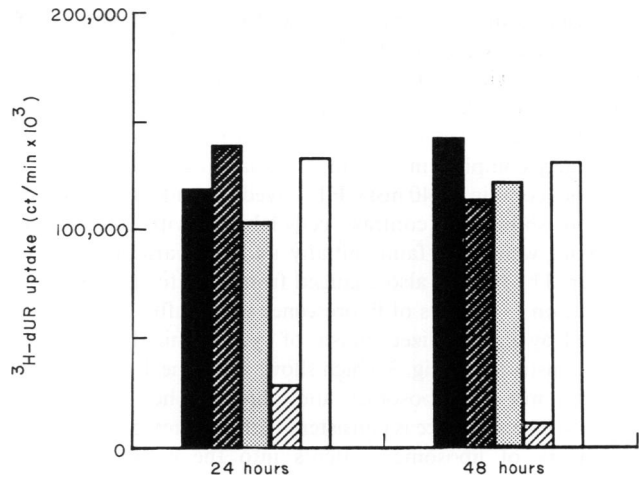


Fig. 4. Effect of liposome encapsulated methotrexate on PHA-lymphoblasts. 2×10^5 PHA-stimulated lymphocytes were cultured in triplicate microtitre wells with; (■) medium alone, (▨) antibody alone, (▩) liposomes containing 20 mM methotrexate, (▩) antibody plus protein A bearing liposomes containing 20 mM methotrexate, and (□) antibody plus protein A bearing liposomes containing buffer (Ab+SUV-PA). The final concentration of antibody was 5 μg/ml and that of liposome-encapsulated methotrexate was 750 nM. 1 μCi of ^{3}H-dUr was added to each well for the last 6 h of culture.

highly quenched (98·4%). As incubation with 200 mM CF liposomes at 37°C proceeded, the increase in the number of fluorescent cells was therefore due to unquenching of liposomes. This is inconsistent with the shedding hypothesis and suggests internalization of CF followed by cytoplasmic dilution and unquenching of fluorescence. An alternative explanation is that this increase in cell fluorescence is simply due to loss of CF from membrane bound liposomes without entry of the fluorophore into the cell. This would lead to unquenching of the liposomes and a gradual increase in brightness. However, further analysis of the location of this fluorescence using a mixture of activated papain and DTT (ZZAP) showed this interpretation to be incorrect. PBL were labelled with fluorescent liposomes at 4°C, washed and analysed on the FACS giving the expected positivity of 72% (Table 3). The cells were divided into two fractions which were incubated at 4° and 37°C respectively for 1 h after which they were put on ice and re-analysed. The cells kept at 4°C were unchanged whereas those incubated at 37°C were now 33% positive. Both fractions were treated with ZZAP for 15 min at room temperature, washed at 4°C and re-examined. The fluorescence of cells held at 4°C was reduced to background (6%) whereas the majority of cells which were incubated at 37°C were still positive. The reduction from 33% to 25% presumably results from the removal of residual cell bound liposomes. These data suggest that the liposomes taken up by PBL at 4°C are primarily associated with the membrane while subsequent incubation at 37°C for 1 h allows a substantial number to become internalized and no longer susceptible to enzyme stripping.

UV microscopy

The appearances and behaviour of PBL incubated with liposomes was studied by UV microscopy in an attempt to elucidate the mechanism of internalization. Lymphocytes incubated with

fluorescent antibody undergo a well-recognized pattern of 'patching' and 'capping'. This process was readily observed in PBL labelled with UCHT1 and GαM-FITC at 4°C and placed on a chilled slide. Upon warming of the slide on the miroscope stage, T cells initiated the patching and capping sequence which was largely complete in 20 min. Cells labelled with protein A liposomes containing 40 mM CF behaved in an identical fashion (data not shown). In contrast, cells labelled with 200 mM CF liposomes were very faint initially and on warming became brighter. The pattern also changed from a uniform peripheral distribution to patches of fluorescence which after 20 min was followed by a generalized 'blush' of cytoplasmic fluorescence. This is illustrated in Fig. 3 which shows the same T cell labelled with 200 mM CF liposomes and photographed at 10 min intervals. The sequence is consistent with the previous evidence of delivery of liposome contents into the cytoplasm with subsequent unquenching and it was found much easier to define the intracellular location of liposomally delivered fluorescence than that left after capping of fluorescent second-layer antibody. Moreover the similarity in the patching and capping of cells labelled with 40 mM CF liposomes compared to those labelled by conventional indirect fluorescence suggests that the handling and fate of the different receptor-ligand complexes is the same.

Cytotoxicity of protein A liposomes containing MTX

The ability of protein A liposomes to deliver cytotoxic quantities of a drug specifically to T cells via the CD3 antigen was tested using MTX. A 20 mM solution of MTX was encapsulated assessed by measuring the uptake of the radioactive substrate ^{3}H-deoxyuridine. PBL were stimulated with PHA for 72 h and then incubated with UCHT1 and liposomes. Control cells were PHA lymphoblasts incubated with medium alone, antibody alone, liposomes containing 20 mM MTX but without protein A or antibody with protein A liposomes containing buffer only. It was seen (Fig. 4) that MTX encapsulated in protein A liposomes targeted with UCHT1 caused an 80% inhibition of ^{3}H dUr incorporation at 24 h and 90% inhibition at 48 h. Control cells were not inhibited, in particular those incubated with untargeted liposomes containing MTX. This confirms the specificity of the targeting and the stability of the liposomes which are sufficiently impermeable to prevent non-specific cytotoxicity due to leakage after 48 h in culture.

DISCUSSION

Clinical use *in vivo* of targeted liposomes has been handicapped by problems with reticuloendothelial system uptake, instability in plasma and tumour accessibility (Weinstein, 1984). Manipulation of bone marrow *ex vivo* for transplantation circumvents these obstacles and may be an ideal application for targeted liposomes. As an antigen target, the human T-cell receptor complex is a good potential candidate. It is lineage specific whereas previously studied antigens for liposome uptake by lymphoid cells have been class I and class II MHC (or HLA) antigens (Machy *et al.*, 1982; Huang *et al.*, 1983). These are expressed by other cell types and would therefore be inappropriate for clinical usage. In addition, there is some evidence that following antibody binding, CD3 is rapidly internalized (Kan, *et al.*, 1983) and may therefore be capable of mediating the internalization of attached liposomes. This could be of value in T cell depletion of bone marrow and may avoid some of the difficulties of current methods which employ bulk complement lysis techniques.

Our studies have shown that protein A liposomes attach to T cells in a manner which is rapid and entirely antibody dependent. The intracellular delivery of liposome contents is more difficult to demonstrate conclusively by a single technique. However, flow cytometry combined with UV microscopy and an enzyme stripping method clearly showed that surface-bound liposomes can deliver their contents into the cell cytoplasm within 30 min at 37°C. When MTX is encapsulated in the liposomes the inhibition of cell growth confirms specific intracellular delivery of liposome contents and demonstrates the feasibility of delivering cytotoxic quantities of drug via the CD3 pathway.

In their study of the fate of CD3 on human T cells after antibody-induced modulation, Kan *et al.* showed rapid internalization of the antigen without shedding into the extracellular medium. This was done using methods including surface iodination of the membrane component, precipitation with antibody and analysis by SDS-PAGE. For the purpose of establishing the fate of surface membrane components after antibody binding, we suggest that the use of fluorescent liposomes and flow cytometry offers an alternative method which is both simple and quick. By indicating which receptors are rapidly endocytosed the results could also have predictive value for the utility of a particular antigen as a receptor for cytotoxic liposomes.

MTX has several desirable properties with respect to encapsulation of cytotoxic compounds in antibody-directed liposomes. It is highly water-soluble, stable for many months in aqueous solution, is active clinically particularly against lymphoid malignancies and, encapsulated in liposomes, has proved effective in previous *in vitro* studies. However, it is active only against dividing cells, so unstimulated or malignant cells in G_0 of the cell-cycle are unaffected. This is shown in the present experiments by the need for prior lectin-stimulation of lymphocytes which is obviously impractical from the point of view of T cell depletion of bone marrow. Nevertheless, the absence of non-specific cytotoxicity to untargeted cells, holds out the possibility that stem cells and hence bone marrow engraftment would be similarly unaffected. This has prompted our investigation of agents which are rapidly cytotoxic to both resting and dividing cells. Encapsulated in liposomes and combined with the highly specific targeting conferred by monoclonal antibodies, such an agent may make feasible not only T cell depletion but the purging from autologous bone marrow of any unwanted or contaminating cells, e.g. carcinoma or residual haematological malignancy.

The attachment of the liposomal bilayer of protein A (or similar ligand for immunoglobulin) as opposed to the targeting antibody itself has several advantages. It is more economical with the targeting antibody since direct coupling of liposome and antibody requires much larger and purer quantities of the latter. Direct coupling commits those liposomes to a single antigen target whereas a single batch of protein A liposomes can be used against any antigen for which a suitable antibody is available. This offers the possibility of using a cocktail of antibodies against several antigens on the same cell thereby increasing the cytotoxic potential of a particular liposome dose. In a similar manner several low density antigens which individually may not be sufficiently numerous to mediate antibody-

targeted liposomal cytotoxicity, may be combined to achieve this effect using the relevant panel of antibodies and a single batch of liposomes.

In conclusion, antibody-targeted liposomes carrying fluorescent, cytotoxic or metabolically active compounds have a useful role in the study of interactions between surface membrane components and the cytoplasmic compartment which could be therapeutically exploited in the *ex vivo* manipulation of bone marrow.

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