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The use of antibody-coated liposomes as a target cell model for antibody-dependent cell-mediated cytotoxicity

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SUMMARY

A simple model system was developed for antibody-dependent cell-mediated cytotoxicity (ADCC) using antibody-coated synthetic membranes (liposomes) as a target cell model. A synthetic hapten, dinitrophenyl-phosphatidylethanolamine (DNP-PE), was incorporated into fluorophore-quencher-loaded liposomes and the latter were coated with pure anti-DNP antibodies. Normal spleen lymphocytes were capable of binding and subsequently lysing these liposomes. This process is dependent upon the presence of an intact (Fc-containing) IgG molecule and independent of exogenous serum complement. The effector lymphocytes are nylon non-adherent, devoid of Thy-1 antigen and present in nude mice, suggesting an identity with K (Fc receptor positive) lymphocytes. These studies indicate that liposomes may be used as a model to study the requirements for the binding and lysis of target cells in this cell-mediated cytotoxic system.

INTRODUCTION

One of the fundamental properties of the immune system is its capacity to recognize, bind and kill (lyse) a wide variety of target cells. These processes may be mediated either by humoral systems or, alternatively, by the direct action of lymphoid cells. The former mechanism requires the participation of complement components activated by the classical or the alternative pathway. In cell-mediated cytotoxic reactions two major mechanisms have been described: T-cell mediated cytolysis and antibody-dependent cell-mediated cytotoxicity (ADCC). In the latter process, the effector lymphocytes have been shown to interact with antibody-coated target cells and subsequently cause their lysis without the addition of any exogenous complement (Brier, Chess & Schlossman, 1975; Perlmann & Perlmann, 1970; Yust *et al.*, 1975). The effector cells, denoted 'null' cells or K-lymphocytes, have been shown to have a potent Fc receptor and to be devoid of Thy-1 antigen or surface IgG (Greenberg *et al.*, 1973; Melewicz *et al.*, 1977; Wisløff, Frøland & Michaelson, 1974).

In order to elucidate the mechanism of these different cytotoxic reactions (primarily complement-mediated cytotoxicity), simple model systems have been developed. Most of these systems employ antigen-containing lipid vesicles as target cell models. The advantage of such synthetic targets is that they can be prepared in a well-defined manner and manipulated in composition. Such systems have helped elucidate some of the requirements for the cytolytic process, such as extent of exposure of the hapten, charge distribution, membrane fluidity, etc. (Alving, 1976; Kinsky & Nicoletti, 1977; Humphries & McConnell, 1975; Brulet & McConnell, 1976).

Very limited information is available concerning a simple target cell model for cell-mediated cytotoxic

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systems. T-cell killing requires, most probably, a highly complex recognition system (Shearer, Rehn & Garbarino, 1975; Zinkernagel & Doherty, 1977). ADCC, on the other hand, appears to proceed by means of a less complex recognition mechanism, and is thus more amenable to a model system.

A system for the direct and continuous monitoring of liposome lysis has recently been developed (Smolarsky *et al.*, 1977; Geiger & Smolarsky, 1977). This system uses a complex of the water soluble fluorophore 1-amino-naphthalene-3,6,8-trisulfonic acid (ANTS) and the quencher bispyridinium-p xylene (DPX). This complex, when entrapped in liposomes, has a very low fluorescence, whereas, upon lysis of the liposome, the quencher is diluted in the extra-liposomal volume, the quench abolished and a fluorescent signal generated. The liposomes remain stable for long periods (over a week) without substantial leakage of the fluorophore; this represents a distinct experimental advantage over a variety of other intraliposomal markers. This system has been used for the study of the kinetics of complement-mediated lysis (Smolarsky *et al.*, 1977), as well as for an immunochemical quantitative determination of lipid antigens (Geiger & Smolarsky, 1977).

In the present study we have used such fluorophore-quencher loaded liposomes as target cell models for the study of ADCC.

MATERIALS AND METHODS

Effector cells. Lymphocytes were isolated from the spleens of non-immune C57Bl/6 mice (2–4 months old). The spleens were removed and the cells suspended in RPMI 1640 medium (Microbiol. Assoc., USA). The cells were washed with RPMI medium and separated by Ficoll–Hypaque (Sigma, USA) density gradient centrifugation (Böyum, 1968; Schreiber *et al.*, 1975). The mononuclear cells were recovered from the interphase and washed twice with Hanks's balanced salt solution (HBSS). Over 95% of the cells were morphologically lymphoid when stained with Wright's Giemsa and examined by light microscopy, and 3% or less were phagocytic (determined by uptake of rhodamine-labelled *E. coli*). Spleen cells were similarly isolated from nude mice (with BALB/B background). Nylon-wool column fractionation of spleen lymphocytes was carried out by established methods (Julius, Simpson & Herzenberg, 1973). Greater than 75% of the nylon non-adherent cells were found to be Thy-1 positive by both cytotoxic and immunofluorescent assays. None of the media used throughout this study contained phenol-red indicator so as to avoid interference in the fluorescent measurements.

Targets. Multilamellar liposomes containing ANTS-DPX in their inner aqueous volume were prepared by vigorous mixing with a Vortex mixer at top speed as described previously (Smolarsky *et al.*, 1977; Geiger & Smolarsky, 1977). Sonicated liposomes were prepared similarly except that the lipid suspension was sonicated for 4×30 sec (with 15 sec intervals), using a Branson B12 sonicator, equipped with a titanium microprobe. The small liposomes were then separated from large liposomes, as well as from unentrapped fluorophore, by gel filtration on a Sepharose 6B column and the final liposome concentration was adjusted to 4·0 mg of total lipids per ml. The standard lipid constituents used throughout the experiments have been described previously (Geiger & Smolarsky, 1977). The composition of the standard lipid mixture used for preparation of liposomes (mix A) consisted of phosphatidylcholine (PC) (from egg yolk, Lipid Products, U.K.); cholesterol (Sigma, USA); dicetylphosphate (Sigma, USA) and α-tocopherol (Assia Lab, Israel), in a molar ratio of: 4 : 3 : 0·1 : 0·5, respectively.

Dinitrophenyl (DNP) modified liposomes included, in addition, 10% (by lipid weight) DNP phosphatidylethanolamine. DNP-PE was synthesized essentially according to Kinsky (1972) and was added to mix A in the organic solvents, prior to liposome preparation.

Antibodies. Pure antibodies to DNP were evoked in rabbits by three repeated injections of 2·0 mg of DNP-bovine serum albumin (BSA) in Freund's complete adjuvant at 10 day intervals. The antibodies were isolated by affinity chromatography on a column of Sepharose-bound Σ-DNP-lysine. Guinea-pig antibodies towards DNP were prepared by injections of DNP-Keyhole-limpet haemocyanin (KLH) (1·0 mg in an equal amount of Freund's complete adjuvant, at 14 day intervals) and isolated by affinity chromatography using DNP-BSA coupled to Sepharose 4B. The rabbit anti-DNP had an average affinity (Ko) towards Σ-DNP-lysine of about $5\times 10^{8}\text{M}^{-1}$ (determined by fluorescent quenching titration) (Eisen & Siskind, 1964). The guinea-pig antibodies had an extremely high affinity of over 10^{10}M^{-1}.

$F(ab')_2$ was prepared from the rabbit anti-DNP antibodies by pepsin digestion (Nisonoff *et al.*, 1960). The Fab′ fraction was obtained by reduction and alkylation of the $F(ab')_2$ fragment (Nisonoff *et al.*, 1960). The affinity of the fragments was not different from that of the intact antibody molecule.

Rabbit anti-mouse IgG was prepared by ammonium sulfate precipitation (40% saturation at 4°C) followed by DEAE cellulose chromatography (eluted in 10 mM sodium phosphate buffer, pH 8·0). These antibodies were conjugated with fluorescein isothiocyanate (FITC) following the method of Brandtzaeg (1973). Antibodies to Thy-1 antigen were kindly provided by Dr P. Lonai of the Weizmann Institute of Science. The activity of the complement components C1 and C2 was determined by effective molecule titration (Mayer, 1961; Schreiber *et al.*, 1976).

Optical measurements. Adsorbance was routinely measured using a Zeiss PMQ II spectrophotometer. Fluorescence measurements were performed and recorded in a Perkin-Elmer spectrofluorometer Model MPF 4IA.

RESULTS

Binding of antibody-coated liposomes to mouse spleen lymphocytes

For the quantitative determination of cell-bound liposomes, lymphocytes (5×10^6, unless otherwise specified) were incubated with the liposomes (40 μg total lipids including 4·0 μg DNP-PE) in a vol. of 100 μl HBSS. For determinations of Fc-mediated binding, the liposomes were pre-incubated with sub-agglutinating concentrations of anti-DNP antibodies prior to the addition of the cells. Following a 90 min incubation period at 37°C, the cells were washed twice with HBSS (120 ***g*** for 10 min) at room temperature and the cell-associated fluorescence measured, either without any further treatment or after the addition of Triton X-100. The results are summarized in Table 1. As can be seen, DNP-containing liposomes sensitized by specific antibodies were bound to a significant extent to normal spleen lymphocytes. This binding was specific and was dependent upon the presence of an intact IgG molecule; it could be inhibited by blocking the antibody-combining site with DNP-BSA and did not occur in liposomes devoid of DNP. The fluorophore bound to the cell was apparently still entrapped inside intact liposomes, as observed by the ratio between the fluorescence in the presence or absence of Triton X-100.

Target lysis

The capacity of the lymphocytes to lyse the bound liposomes was studied as follows: 5×10^6 lymphocytes were incubated with antibody-coated DNP-PE liposomes and washed as described above. The cells were then incubated in HBSS supplemented with 5% heat-inactivated (56°C × 30 min) foetal calf serum (FCS) in a humid atmosphere of 5% CO_2 and 95% air at 37°C. At different time points samples were withdrawn and their fluorescence with or without Triton X-100 measured. The fluorescence obtained in the absence of detergent represents the extent of liposome lysis and release of the fluorophore, whereas the fluorescence in the presence of Triton X-100 measures the total fluorescence (cell associated + released). The results, expressed as the percentage of release out of the total bound fluorescence obtained in three independent experiments, are depicted in Fig. 1. Progressive liposome lysis was apparent, reaching a maximal value after 8–10 hr of incubation. The values of maximal release at the plateau ranged from 33–50% of the total liposomes bound. The released fluorophore was found exclusively in the medium, as determined by centrifugation (1500 ***g*** for 15 min) followed by fluorescence measurement of the supernatant and the pellet. The process of lysis most probably required direct

TABLE 1. Binding of DNP-PE liposomes by spleen lymphocytes

Spleen lymphocytes	Liposomes	Antibody preparation	Cell-bound fluorescence (arbitrary units)*
5×10^6	10% DNP-PE, 90% Mix A	0	3
5×10^6	10% DNP-PE, 90% Mix A	anti-DNP, 80 μg/ml	40
5×10^6	10% DNP-PE, 90% Mix A	anti-DNP, 40 μg/ml	23
5×10^6	10% DNP-PE, 90% Mix A	anti-DNP, 20 μg/ml	17
5×10^6	10% DNP-PE, 90% Mix A	anti-DNP, 60 μg/ml $F(ab')_2$	0
5×10^6	10% DNP-PE, 90% Mix A	anti-DNP, 60 μg/ml Fab′	0
5×10^6	10% DNP-PE, 90% Mix A	anti-BSA, 100 μg/ml	4
5×10^6	10% DNP-PE, 90% Mix A	anti-DNP, 80 μg/ml† + 100 μg/ml DNP-BSA	5
0	10% DNP-PE, 90% Mix A	anti-DNP, 80 μg/ml	0
1×10^6	10% DNP-PE, 90% Mix A	anti-DNP, 80 μg/ml	7
5×10^5	10% DNP-PE, 90% Mix A	anti-DNP, 80 μg/ml	0
5×10^6	100% Mix A	anti-DNP, 80 μg/ml	0

* Determined after addition of 10 μl of 15% Triton X-100.

† Antibodies pre-incubated with DNP-BSA for 30 min at 37°C.

Mix A buffer: 0·1M Tris buffer, pH 7·7 (Smolarsky *et al.*, 1977).

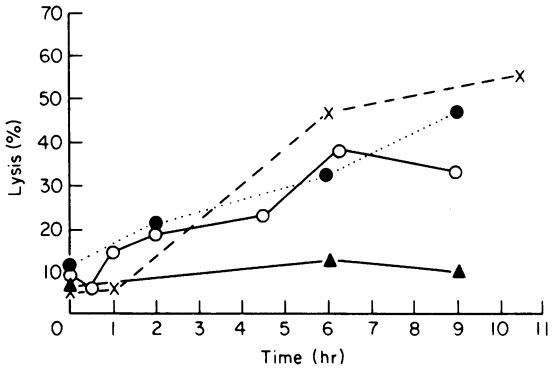

FIG. 1. Lysis of lymphocyte-bound IgG-coated liposomes. Three experiments are illustrated. (▲——▲) Indicates the lysis of liposomes (not coated with IgG) by lymphocytes.

contact between the lymphocytes and the liposomes, as the cell-free culture medium did not cause any lysis of antibody-coated DNP-PE liposomes. Control experiments indicated that liposomes unassociated with lymphocytes remained intact under the culture conditions and that DNP-PE liposomes which were not coated with IgG were not significantly lysed when incubated in the presence of lymphocytes (Fig. 1). It should be mentioned that the anti-DNP antibody preparation used here, as well as the incubation medium and the heat-inactivated FCS, contained $< 1\%$ detectable functional C1 or C2, as assessed by effective molecule titration, and no active properdin factor B (Alper, Goodkofsky & Lepour, 1973).

Characterization of the effector lymphocytes

In an attempt to characterize the cell populations responsible for the binding and lysis of the liposomes, partial fractionation of the spleen cells was carried out. The total spleen lymphocyte population (after Ficoll–Hypaque gradient centrifugation) was incubated at 37°C on plastic dishes. This treatment reduced the number of phagocytic cells (determined by phagocytosis of *E. coli*) from about 3% to less than 0·5%. Fractionation of the cells on nylon wool columns resulted in two distinct populations of cells. The nylon non-adherent population consisted of 75–80% Thy-1-positive cells and less than 5% IgG-positive cells (determined by direct immunofluorescence with FITC-conjugated antibodies towards mouse IgG). The nylon-adherent cell population consisted of 85–90% IgG-positive cells and only about 2% Thy-1-positive cells. In addition, T cells were depleted from a normal spleen lymphocyte population by anti-Thy-1 in the presence of guinea-pig complement *in vitro*, after which the cells were extensively washed. Samples containing 5×10^6 cells of the Thy-1-depleted and control populations were examined for binding of the antibody-coated liposomes. The results are shown in Table 2. The experimental procedure was the same as described for Table 1. The binding capability was found to reside in the plastic non-adherent and the nylon non-adherent populations. The nylon adherent lympho-

TABLE 2. Binding of IgG-coated liposomes to different lymphocyte populations

Spleen lymphocytes (5×10^6 cells)	Concentration of anti-DNP (μg/ml)	Cell-bound fluorescence (arbitrary units)
Unfractionated	80	36
Plastic non-adherent	80	40
Nylon non-adherent	0	0
Nylon non-adherent	80	19
Nylon adherent	80	1
Nude mice (unfractionated)	80	30

cytes had diminished binding capacity. Unfractionated spleen cells of nude mice, as well as Thy-1-depleted normal spleen lymphocytes (not shown), were both effective in binding the antibody-coated liposomes. It was verified in all cases that the binding required the presence of intact antibody on the liposomes.

Further incubation of the cells with the bound liposomes for an additional period of 8 hr resulted in 30–60% liposome lysis for all the liposome-binding populations of lymphocytes studied.

DISCUSSION

The experiments described above indicate that liposomes may be used as a model for the study of the binding and subsequent lysis of target cells in ADCC. The cells involved in this process were shown to be nylon non-adherent, non-phagocytic and to be devoid of Thy-1 antigen. This is in agreement with the current concept that K-cells (or null cells) which have Fc-receptors on their surface are the predominant population active in ADCC (Greenberg *et al.*, 1973; Melewicz *et al.*, 1977). The binding is dependent upon antibody concentration and is maximal with an antibody concentration of 80 μg/ml (depending on the quality of the antibody preparations). Higher concentrations (>160 μg/ml) resulted in agglutination and the formation of large liposome clumps. The use of guinea-pig antibodies with a very high affinity ($Ko > 10^{10} M^{-1}$) did not point to any basic difference, except for the fact that a (two- to four-fold) lower concentration of antibody was required for optimal binding.

Some recent attempts have been made to use lipid bilayers (Henkart & Blumenthal, 1975) or liposomes (Juy *et al.*, 1977) as target models in ADCC. In the latter case, Fc-receptor-bearing cells from mouse spleen were observed to specifically lyse liposomes containing cardiolipid in their membranes and coated with antibodies to that antigen. The results described above show that antibody-dependent cell-mediated liposome lysis can take place in a highly defined system consisting of the synthetic antigen DNP, purified antibodies and fluorophore-quencher loaded liposomes in the absence of complement. The applicability of this model to additional antigenic systems was also tested. We observed that liposomes containing incorporated gangliosides (GM_2 and GM_1) and coated with the respective antibodies are amenable for significant binding and subsequent lysis by normal mouse lymphocytes in a reaction at least as efficient as that observed with the DNP system.

In our present study sonicated liposomes were used, though multilamellar vesicles, prepared using a Vortex mixer, were as effective. The main difficulty in the use of multilamellar liposomes was the complete separation of cell-bound from unbound liposomes. The unbound sonicated liposomes could be efficiently removed by a low speed centrifugation (75 ***g*** for 10 min).

All the lymphocyte populations examined which exhibited antibody-mediated liposome binding, were capable of lysing the liposomes upon additional incubation. The lysis was time-dependent and reached a value of up to approximately 50% of the bound vesicles within 8–10 hr. At the end of the incubation period, the viability of the cells declined from over 95% to about 60% (determined by trypan blue exclusion). It is possible that arrest in liposome lysis at 8–10 hr results, at least in part, from this decrease in the number of viable effector cells.

Macrophages also possess Fc receptors on their surface (LoBuglio, Cotran & Jandl, 1967) and are capable of binding antibody-coated liposomes and subsequently internalizing them (unpublished observations). The involvement of macrophages in the lytic process described here was discounted, since (a) the number of macrophages in the spleen suspension tested was too low to elicit significant binding (as determined in experiments with purified macrophage cultures); (b) the activity resided in the plastic and nylon non-adherent population, and (c) the ANTS fluorescence was recovered from the cell-free culture medium, whereas the fluorescence of liposomes removed by macrophages remains intracellular for extended periods.

The major conclusion of this study is that simple lipid vesicles, coated with antibodies, may function as targets in ADCC. Thus, this process does not require an interaction with any specific proteins on the target cell surface other than IgG and it probably operates by direct damage to the target cell membrane. In these respects, the mechanism of ADCC resembles that of complement-mediated lysis and is

clearly different from T-cell killing. In the latter a complex recognition system is operative, requiring the presence of both the proper antigens and histocompatibility antigens on the surface of the target cell (Shearer *et al.*, 1975; Zinkernagel & Doherty, 1977).

The detailed mechanism of cytotoxicity in ADCC is not yet understood. However, it is anticipated that further studies using simple lipid vesicles which may be readily manipulated with respect to their composition, charge, size and fluidity, may shed light on the mechanism of the lytic process at the molecular level.

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