Biochimica et Biophysica Acta, 509 (1978) 272—288

BBA 78012

ANTIBODY-MEDIATED TARGETING OF LIPOSOMES

BINDING TO LYMPHOCYTES DOES NOT ENSURE INCORPORATION OF VESICLE CONTENTS INTO THE CELLS

JOHN N. WEINSTEIN, ROBERT BLUMENTHAL, SUSAN O. SHARROW [a] and PIERRE A. HENKART [a]

Laboratory of Theoretical Biology, National Cancer Institute, Bldg. 10, Room 4B56, N.I.H., and [a] Immunology Branch, National Cancer Institute, N.I.H., Bethesda, Md. 20014 (U.S.A.)

(Received August 8th, 1977)
(Revised manuscript received December 15th, 1977)

Summary

Small sonicated lipid vesicles containing the water-soluble fluorescent dye 6-carboxyfluorescein were formed from dioleoyl phosphatidylcholine and the antigenic lipid *N*-dinitrophenylaminocaproyl phosphatidylethanolamine. When these vesicles were incubated with trinitrophenyl-modified human lymphocytes and divalent anti-trinitrophenyl antibody, the antibody bound 5000 to 15 000 vesicles to each cell. Binding was detected by fluorescence microscopy and quantitated by fluorometry and flow microfluorometry. Binding was three times greater with $F(ab')_2$ fragments than with the whole antibody and, as expected, was almost absent with the monovalent F(ab') fragments. It was also absent or greatly reduced, (i) with control immunoglobulin G, (ii) in the presence of excess soluble trinitrophenyl hapten, or (iii) if hapten was omitted from either cells or vesicles. It was unaffected by sodium azide and 2-deoxy-D-glucose but was markedly decreased at 3°C. It was not reversed by incubation at 3°C with excess trinitrophenyl lysine.

Self-quenching of the fluorescence of 6-carboxyfluorescein was used to distinguish between release of vesicle contents into the cells and simple binding of intact vesicles (Weinstein, J.N., Yoshikami, S., Henkart, P., Blumenthal, R. and Hagins, W.A. (1977) Science 195, 489—491). Antibody-mediated binding led to little or no increase over spontaneous background levels in the amount of vesicle contents released into the lymphocytes.

Abbreviations: Medium I, 167 mM NaCl and 7 mM KCl, buffered to pH 7.4 with 10 mM HEPES; Medium II, Hank's balanced salt solution with 10% heat-inactivated fetal calf serum, buffered to pH 7.4 with 10 mM HEPES. F(ab'), $F(ab')_2$, monovalent and divalent fragments, respectively, of immunoglobulin G; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; IgG, immunoglobulin G; TNBS, trinitrobenzene sulfonate; Tnp, 2,4,6-trinitrophenyl.

Liposome-cell interactions are attracting widespread attention for two principal reasons: first, they may shed light on similar events occurring in such processes as secretion, phagocytosis and viral infection [1]. Second, liposomes may be useful as vehicles for delivery of materials to cells or tissues [2—4]. Parameters such as the size, charge and fluidity of a liposome determine to some extent the site and mode of its interaction with cell membranes [5—11], but more specific means of "targeting" would certainly be necessary for many of the projected applications, or for physiological vesicles. Targeting of liposomes has recently been studied using lectins and cell surface saccharides [12], aggregated immunoglobulin and surface Fc receptors [13,14], asialoglycoproteins and their receptors on hepatocytes [15], and antibody (non-specifically attached to vesicles) and cell surface antigens [15,16].

We report here the use of IgG antibodies as divalent cross-bridges binding antigenic unilamellar lipid vesicles * to antigenic cells. We asked two questions. Can such binding be accomplished? And, if so, are the contents of the vesicles released into the cell?

Liposomes and their contents can apparently enter cells by phagocytosis after binding to the cell surface [13—15]. In order to focus instead on fusion of the vesicle bilayer and cell plasma membrane as a possible mechanism for transfer of vesicle contents, we chose the human peripheral blood lymphocyte as a target cell. The lymphocyte is not phagocytic, and its incorporation of vesicle contents in the absence of targeting appears to depend largely on fusion, or at least on a metabolically passive process [17—20].

Targeting was accomplished by: (i) incorporating the antigenic lipid *N*-dinitrophenylaminocaproyl phosphatidylethanolamine [21] into small unilamellar dioleoyl phosphatidylcholine vesicles; (ii) modifying the lymphocyte surface with the trinitrophenyl hapten; and (iii) adding anti-Tnp antibody to bind vesicles to the cell (antibodies to Dnp and Tnp cross-react). As reported previously [18], fluorescence self-quenching of the water-soluble dye 6-carboxyfluorescein provides a means of distinguishing between vesicle contents released into a cell and those remaining sequestered in vesicles bound to the cell. We found that thousands of vesicles became firmly attached to the surface of each lymphocyte. However, release of vesicle contents into the cells was not significantly increased above the background levels occurring (probably by fusion) in the absence of targeting. An abstract of this work has appeared elsewhere [22].

Methods

Principle of the method. 6-Carboxyfluorescein resembles fluorescein in spectral properties (excitation maximum at 490 nm; emission maximum at 520 nm), but is more polar than fluorescein and less permeant through bilayers [18]. As with other fluorophores it becomes less fluorescent at high concentration ("self-quenching" or "concentration-quenching") because of energy transfer between neighboring fluorophore molecules. Therefore, vesicles containing 200 mM 6-carboxyfluorescein can be used to measure release of vesicle con-

* The terms 'liposome' and 'lipid vesicle' will be used interchangeably.

tents into a cell with little interference from dye remaining in vesicles bound to (or intact within) the cell; such vesicles contribute only about 2% as much to the fluorescence intensity as they would if their dye were released and diluted into the approx. 10^6-fold larger cytoplasmic space. If cells and bound vesicles are disrupted by detergent, releasing all of the fluorophore into the medium, the final fluorescence indicates total cell-associated dye, and the increase in fluorescence reflects the amount of dye in bound vesicles.

With 10 mM 6-carboxyfluorescein, on the other hand, the fluorescence is only about 40% self-quenched and its quantum efficiency is nearly equal to that of very dilute 6-carboxyfluorescein in the cytoplasmic compartment of a lymphocyte (see Appendix for details); hence, the contribution of a dye molecule to the observed fluorescence is the same whether it remains in a bound vesicle or is diluted into the cell. Table I shows the parameters determined by fluorescence microscopy, fluorometry and flow microfluorometry.

Materials. 6-carboxyfluorescein (Eastman, Rochester, New York) was treated with activated charcoal and recrystallized from approx. 1 : 2 ethanol/water (Hagins, W.A. and Yoshikami, S., unpublished method). Dioleoyl phosphatidylcholine was prepared by R.R. Kurtz (Applied Science Laboratories, Philadelphia). Before use its purity was checked by thin-layer chromatography (precoated silica gel G plates, eluted with $CHCl_3/CH_3OH/H_2O$, 65 : 25 : 4, developed with I_2 vapor). 100 μg samples gave single spots. The fatty acid content was more than 99% oleic, as determined by gas-liquid chromatography. Dnp-caproyl phosphatidylethanolamine obtained from Avanti-Biochemical (Birmingham, Ala), gave a single spot at 25 μg on thin-layer chromatography.

We used the following solutions: medium I, 167 mM NaCl and 7 mM KCl, buffered to pH 7.4 with 10 mM HEPES; medium II, Hank's balanced salt solution with 10% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.), buffered to pH 7.4 with 10 mM HEPES. 2,4,6-trinitrobenzene sulfonate (TNBS) was obtained from Pierce Biochemical (Rockford, Ill.) and ϵ-trinitrophenyl lysine (Tnp-lysine) from ICN Pharmaceuticals (Cleveland, Ohio).

Liposome preparation. We prepared sonicated vesicles by a modification of a method described previously [18]: 25 mg dioleoyl phosphatidylcholine and 1.6 mg Dnp-caproyl phosphatidylethanolamine (to make 5 mol % Dnp-caproyl phosphatidylethanolamine vesicles) were dissolved together in benzene/ethanol (1 : 1) in a glass vial, dried to a thin film under nitrogen, and lyophilized overnight. 4 ml of either 10 mM 6-carboxyfluorescein in medium I or 200 mM 6-carboxyfluorescein in H_2O was added and the lipid vortex-mixed into suspension. The suspension was sonicated at 35°C under nitrogen for 1 h with the micro-tip of a Branson Sonifier, model W-350 (Heat Systems-Ultrasonics, Plainview, N.Y.), at power level 3. Suspensions clarified optically within 5 min. The sonicate was cooled in ice, filtered through a rinsed 0.22 μm Millipore filter, and chromatographed at 3°C on a 26 × 60 mm column of Sephadex G-50 fine (Pharmacia, Piscataway, N.J.) in medium I. Vesicles were eluted in the void volume, and free dye was retarded by the gel. The 6-carboxyfluorescein content of each preparation was determined from fluorometric measurements before and after addition of Triton X-100 detergent [18]. Control vesicles were prepared similarly but without Dnp-caproyl phosphatidylethanolamine.

TABLE I

SYNOPSIS OF EXPERIMENTAL APPROACH AND RESULTS

Technique of analysis	Vesicles contain	Parameter determined	Cell-associated 6-carboxyfluorescein (V_F or V_T) (vesicle-equivs./cell)	
			Targeted *	Control **
Fluorescence microscopy	10 mM carboxyfluorescein	Morphology	patchy rim; also faint diffuse fluorescence	faint diffuse fluorescence only
Fluorometry				
pre-Triton	200 mM 6-carboxyfluorescein	Free intracellular 6-carboxyfluorescein (V_F)	320 ± 30 (2)	260 ± 50 (4)
post-Triton	200 mM 6-carboxyfluorescein	Total cell 6-carboxyfluorescein (V_T)	5450 ± 60 (2)	240 ± 30 (4)
Flow microfluorometry	200 mM 6-carboxyfluorescein	Free intracellular 6-carboxyfluorescein (V_F)	370 ± 10 (2)	290 ± 20 (6)
	10 mM 6-carboxyfluorescein	Total cell 6-carboxyfluorescein (V_T)	4250 ± 130 (3)	270 ± 70 (10)

* Mean ± S.E. (number of experiments in parentheses) of results with Tnp-cells, IgG anti-Tnp, and Dnp vesicles (pooled averages from Tables II and III). See text for conditions of incubations.

** Mean ± S.E. (number of experiments in parentheses) of the controls (pooled averages from Tables II and III).

Vesicles prepared in this way were characterized by quasi-elastic light-scattering (in collaboration with R. Nossal, N.I.H.) and by 6-carboxyfluorescein/phospholipid ratios [18]. For calculations we will use numbers determined by Hauser et al. [23] for 250-Å phosphatidylcholine vesicles: internal volume $2.2 \cdot 10^{-18}$ cm^3, lipid content 4000 molecules. Systematic errors in these estimates would have no effect on the arguments we present.

The liquid-crystalline transition temperature of dioleoyl phosphatidylcholine is −22°C, and the Dnp-caproyl phosphatidylethanolamine was synthesized from egg phosphatidylcholine; hence, both control and haptenated vesicles were in the fluid state. Leakage of 6-carboxyfluorescein was negligible over a period of hours at 3°C; leakage during 30-min incubations with cells in 10% fetal calf serum at 37°C averaged 14%, with no clear difference between the two types of vesicle. At least 93% of the vesicles could be removed from suspension by specific antibody-mediated binding to Dnp-Sepharose (unpublished data), suggesting that Dnp hapten was well-distributed among the vesicles.

Antibody. IgG antibodies to Tnp hapten were prepared from the serum of sheep hyperimmunized to Tnp-keyhole limpet hemocyanin. The antibodies were purified by affinity chromatography on Dnp-lysine Sepharose (elution with 0.1 M acetic acid), followed by gel filtration on Sephadex G-200. $F(ab')_2$ fragments were prepared by pepsin digestion, followed by gel filtration on Sephadex G-150. F(ab') fragments were then made by reduction and alkylation. Hemagglutination titers of the IgG anti-Tnp and $F(ab')_2$ anti-Tnp were the same (on a molar basis). Normal sheep IgG was purchased from Miles Laboratories (Elkhart, Ind.).

Lymphocytes. Human peripheral blood lymphocytes from healthy donors were purified with carbonyl iron and Ficoll-Hypaque [24]. The cells, which included fewer than 1% monocytes, were kept overnight in Eagle's medium with 10% fetal calf serum and re-suspended in medium II prior to use. Tnp-modified lymphocytes were prepared by incubation at 37°C for 15 min with 1 mM TNBS in phosphate-buffered saline at pH 7.4. Such lymphocytes remain viable and have been used extensively for immunological studies. Tnp-modified mouse lymphocytes cap both surface immunoglobulin and H-2 antigens (Schmitt-Verhulst, A.-M., Dickler, H.B., Shearer, G.M. and Henkart, P.A., unpublished), express normal alloantigens [25], and are capable of generating cytotoxic killer cells after in vitro culture [26]. TNBS modifies free amino groups of lymphocyte surface proteins [27] and probably modifies aminolipids as well.

Incubations. Lymphocytes were first incubated with antibody in polystyrene tubes at 37°C for 15 min. Each tube contained approx. $4 \cdot 10^6$ cells in 0.5 ml medium II. The fetal calf serum greatly reduces spontaneous transfer of 6-carboxyfluorescein from vesicles to lymphocytes [28]. 50 μl of vesicle suspension were then added and incubation continued at 37°C for 30 min. The tubes were shaken gently to disperse vesicles and then not agitated again. Incubations were terminated by adding 2.5 ml of iced medium II. The cells were washed twice by low-speed centrifugation with iced medium and then transferred to clean tubes. Fluorescence could be determined at leisure, as there was little change over several hours at 3°C. Vesicles were always in great excess; in no case did

more than 3% of them bind to lymphocytes during an incubation.

Except as otherwise indicated, vesicles and immunoglobulin were added to the incubation media as appropriate, to the following final concentrations:

vesicles containing 10 mM 6-carboxyfluorescein, 0.17 μM 6-carboxyfluorescein, 52 μM lipid

vesicles containing 200 mM 6-carboxyfluorescein: 2.2 μM 6-carboxyfluorescein, 34 μM lipid

whole IgG, F(ab'), F(ab')$_2$, 1.12 μM hapten-combining sites.

Fluorescence microscopy. After washing, cells were sealed under a coverslip in Medium II and examined under fluorescence and phase optics with a Zeiss Universal microscope.

Fluorometry. 100-μl aliquots of vesicle or cell suspension were placed in small cuvettes and fluorescence determined with an Aminco-Bowman fluorometer (Aminco, Silver Spring, Md.) before and after releasing 6-carboxyfluorescein from the cells or vesicles with 5 μl of 20% Triton X-100 detergent. Results were corrected for the volume added and, when significant, for the low background fluorescence of Triton. At the concentration used, Triton does not significantly affect the fluorescence efficiency of 6-carboxyfluorescein.

The fluorescence observed before addition of detergent indicates the number of "vesicle-equivalents" of 6-carboxyfluorescein released on average into a cell (V_F), whereas the fluorescence after detergent addition indicates total cell-associated fluorophore (V_T). The amount of 6-carboxylfluorescein in vesicles bound to the cells (V_V) is the difference between the two ($V_V = V_T - V_F$). V_F, V_T, and V_V represent the number of vesicles per cell whose total 6-carboxyfluorescein contents would be required to produce the observed cell fluorescence. Expressing the results in "vesicle-equivalents" rather than in numbers of molecules permits direct comparison of results from the vesicles containing 200 mM and 10 mM 6-carboxyfluorescein. The term "vesicle-equivalent" is used rather than "vesicle", since the incorporation process might be leaky. If, for example, it were 50% leaky, twice as many vesicles would be required to produce a given fluorescence. The calculations are described in detail in the Appendix.

Readings were normalized by the concentration of cells, as counted in a hemocytometer. Tnp-modification of cells and the presence of vesicles made no discernible difference in cell viability; after incubation, 1—3% of cells were positive for Trypan blue entry. However, the combination of Tnp-cells and anti-Tnp, with or without haptenation of vesicles, resulted in 8—10% Trypan blue positive cells.

Flow microfluorometry. We used a FACS-1 (Becton-Dickinson Laboratory, Mountain View, Calif.). Analytical use of the instrument has been described by Loken and Herzenberg [29]. Cells were passed in a rapidly flowing stream of medium through the beam of an argon laser (tuned to the 488-nm line and focused with a 32-mm focal length spherical lens). The fluorescence (or light-scattering) associated with each individual cell registered in a multi-channel analyzer, and the results for a run of $1 \cdot 10^4$ to $1 \cdot 10^5$ viable cells appeared in a frequency histogram giving the distribution of cell fluorescences within the population (see Figs. 1 and 2). A criterion based on light-scattering was used to exclude non-viable cells from consideration [30]. We calibrated the FACS by comparing computed means of cell fluorescence with fluorometric readings on the same suspensions of cells.

Results

We apply the term "targeted" to incubations in which antibody-mediated binding would be expected, i.e., those containing Tnp-lymphocytes, Dnp-vesicles and divalent anti-Tnp; incubations lacking one or more of the above specific components are called "controls".

Fluorescence microscopy. Fig. 1a is a fluorescence micrograph of lymphocytes from a targeted incubation with IgG anti-Tnp and vesicles containing 10 mM 6-carboxyfluorescein. Most of the fluorescence appeared as a patchy rim at the margin of the cell, and focusing up and down confirmed that most of the dye was localized at the cell surface. This clearly represented dye in intact vesicles since incubations with free 6-carboxyfluorescein and vesicles containing only buffer yielded no surface fluorescence. We saw no capping of the vesicles. Fig. 1b, a phase micrograph of the same field, indicates that all cells in the population showed this pattern of fluorescence. In contrast, there was little fluorescence in any of the controls. The appearance of cells from the targeted system was distinctly different from that seen in previous studies under conditions which favored transfer of 6-carboxyfluorescein into the cells [18]; in the

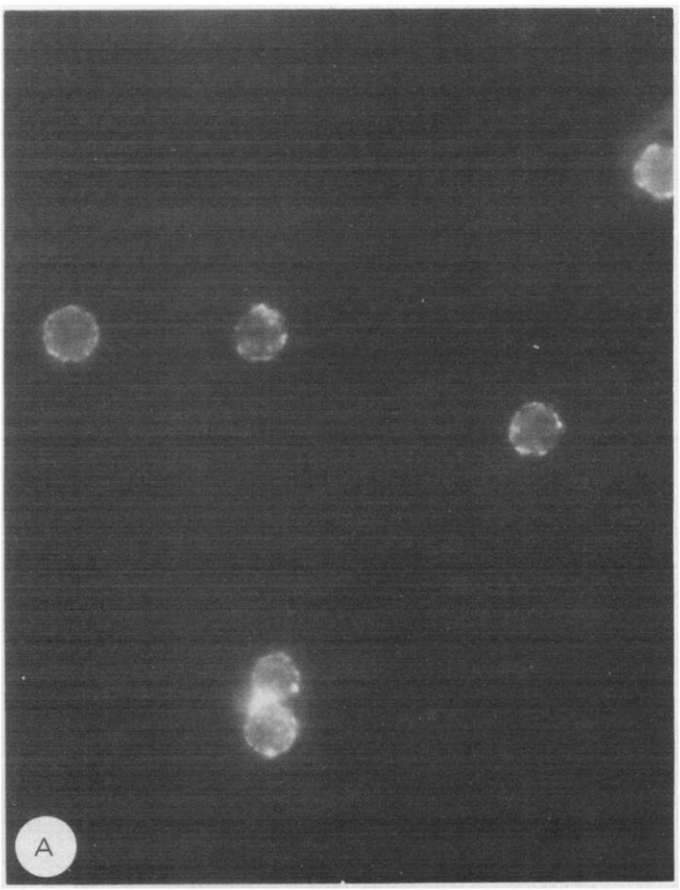

Fig. 1A.

latter case a relatively uniform cytoplasmic staining (with somewhat less intensity in the nucleus) was observed, indicating release of dye into the cell.

Fluorometry. Table II shows the results of fluorometric readings on lymphocytes incubated with vesicles containing 200 mM 6-carboxyfluorescein. Targeted and control systems differed little in V_F (an average of 320 vesicle-equivalents in the former, 260 in the latter; see Table I). Even the small apparent difference may have been due to residual fluorescence of bound vesicles in the targeted case, or to slight leakage from those vesicles during measurement.

After disruption of vesicles and cells with Triton, the fluorescence of lymphocytes from the targeted system increased 17-fold. Total cell-associated 6-carboxyfluorescein (V_T) averaged 5500 vesicle-equivalents, and there were, therefore, approx. 5200 intact vesicles (V_V) bound to the cell surface (ignoring a 14% correction for vesicle leakage described in Appendix). Preliminary kinetic studies indicate that vesicle binding has slowed but not ceased by the

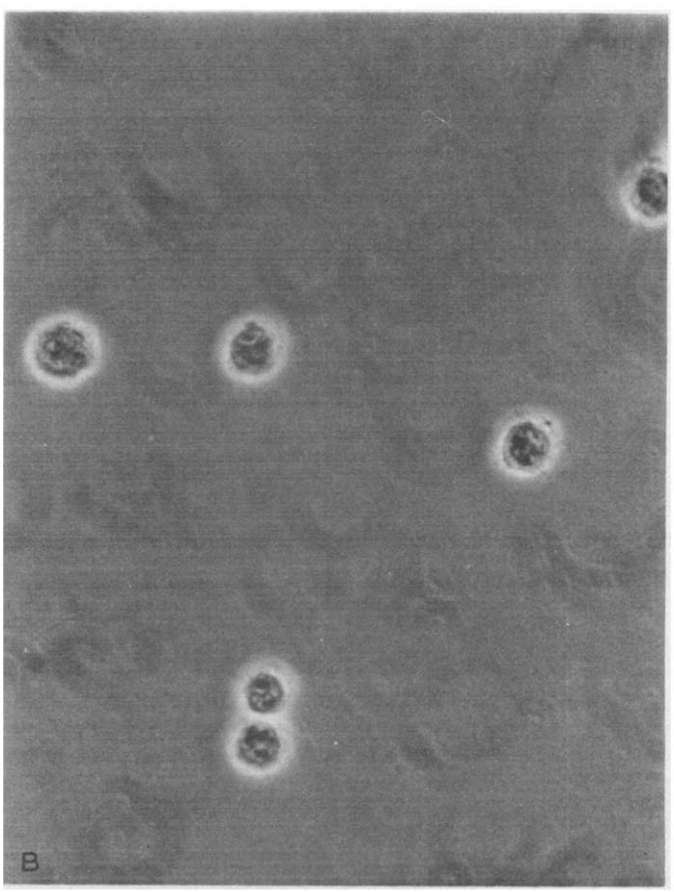

Fig. 1. Tnp-modified human lymphocytes incubated with IgG anti-Tnp and Dnp-vesicles containing 10 mM 6-carboxyfluorescein (targeted system). (A) Fluorescence micrograph showing most of the fluorescence in a "patchy rim" at the cell surface. (B) The same field under phase optics. It demonstrates that all of the cells in the population fluoresce. The incubation medium contained 260 μmol lipid and 0.85 μmol 6-carboxyfluorescein per liter. (×1000)

TABLE II

FLUOROMETRIC STUDIES OF LYMPHOCYTES FROM INCUBATIONS WITH VESICLES CONTAINING 200 mM 6-CARBOXYFLUORESCEIN

Total cell-associated dye (V_T) was much greater in the targeted system than in the controls, but there was little difference between them in the amount of dye released from vesicles into the cells (V_F). V_T and V_F were determined, as explained in the text, from measurements of fluorescence before and after addition of Triton, respectively. The appearance in two cases that $V_F > V_T$ probably arises from measurement uncertainty. See text for conditions of incubation.

Antibody	Tnp on cells	Dnp on vesicles	Cell-associated dye (vesicle-equivs./cell)	
			Total (V_T)	Released into cell (V_F)
Targeted				
IgG anti-Tnp	+	+	5500	340
			5400	290
Control				
normal IgG	+	+	160	200
normal IgG	—	—	200	330
IgG anti-Tnp	—	—	300	200
			380	240

end of the 30-min incubation period, that increasing the number of cells in the incubation has no effect on binding per cell, and that increasing the concentration of Dnp-vesicles or anti-Tnp increases the binding.

In contrast with the targeted incubations there was little, if any, increase in fluorescence of the controls after addition of Triton. The data are not precise enough to rule out a low level of vesicle binding, but there was clearly none on a scale comparable to that in the targeted system.

Repetition of these studies (and of those reported in Figs. 3—6) on other days with different batches of vesicles and cells yielded the same pattern of results, though the absolute numbers were variable by about a factor of two.

Anti-Tnp caused some aggregation of Tnp-lymphocytes: 13—23% of cells appeared in clumps of more than two cells, as compared with 0—6% for the other controls. Suspensions of Dnp-vesicles did not become turbid upon exposure to anti-Tnp, but the possibility of small clumps was not excluded.

Flow microfluorometry

(i) Total cell-associated 6-carboxyfluorescein (V_T). Fig. 2 shows frequency histograms obtained from the FACS using 10 mM 6-carboxyfluorescein vesicles in the targeted system with IgG anti-Tnp (A) and in a control with normal IgG (B). Much more fluorophore was associated with cells in the former (V_T = 1400 vesicle-equivalents/cell) than in the latter (V_T = 200 vesicle-equivalents/cell). In the targeted case almost all of the cell signals appeared in a single broad fluorescence peak.

To test whether specific binding is confined to hapten-modified cells, we mixed 50% Tnp-modified and 50% untreated cells in the same incubation tube, with anti-Tnp and haptenated vesicles containing 10 mM 6-carboxyfluorescein. Fig. 3 shows the resulting bimodal distribution of cell fluorescences; such distributions were never seen in incubations with one cell type, and the two distinct

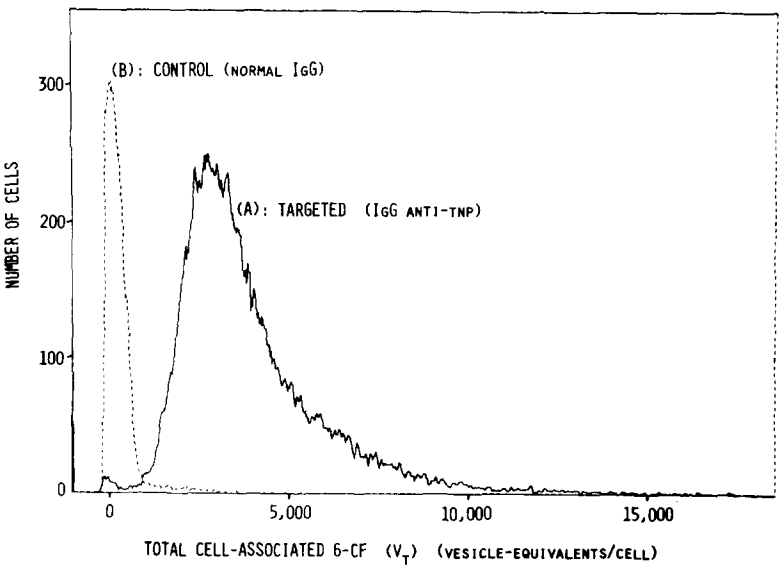

Fig. 2. Frequency distributions of total cell fluorescence obtained by flow microfluorometry. (A) Tnp-modified lymphocytes incubated with IgG anti-Tnp and vesicles containing 10 mM 6-carboxyfluorescein (targeted system); (B) an incubation identical except that normal IgG was substituted for IgG anti-Tnp (a control). In each case, almost all of the cell signals fell into a single peak (though sub-populations could be hidden in the shoulders of those peaks). Fewer than 1% of cells gave off-scale signals. By the criterion of light-scattering, 16% of cells in A and 6% in B were dead or dying; if these were included in the computation, the values of mean fluorescence decreased by a few percent.

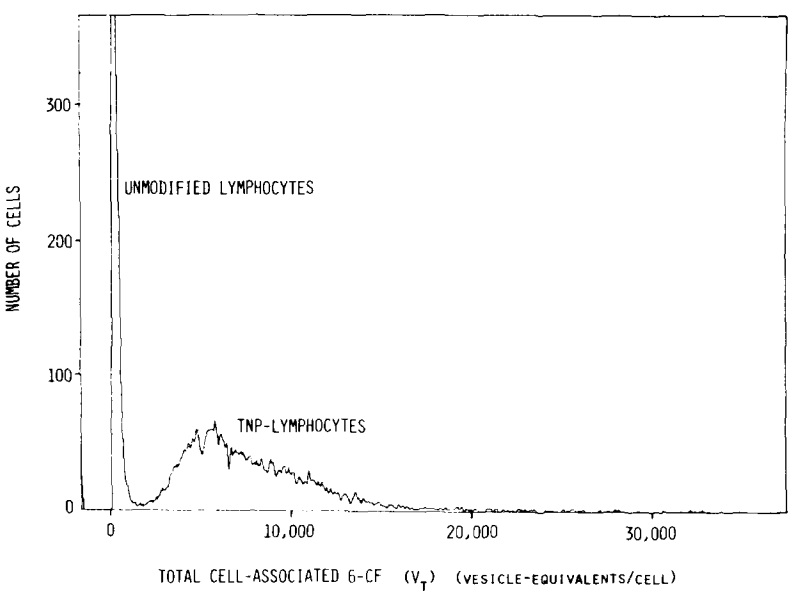

Fig. 3. Population distribution of the fluorescences obtained by flow microfluorometry on Tnp-modified and unmodified lymphocytes mixed together in an incubation with IgG anti-Tnp and Dnp-vesicles containing 10 mM 6-carboxyfluorescein. The bimodal distribution indicates that ability to bind vesicles was not transferred from Tnp-modified to unmodified cells. The incubation medium contained 58 μmol of lipid and 0.19 μmol 6-carboxyfluorescein per l.

populations contained 49 (lower fluorescence) and 51% (higher fluorescence) of the cells. Thus the capacity to bind vesicles was not transferred from haptenated to unmodified cells.

The next-to-last column in Table III shows the fluorescence of cells incubated with vesicles containing 10 mM 6-carboxyfluorescein. The targeted system with whole IgG appeared 16 times as fluorescent as the average of the controls, in good agreement with the fluorometry results in Table II. It should be noted that the values obtained for the various controls were not much above the background subtracted for the fluorescence of untreated cells (220 vesicle-equivalents), so differences among them must be interpreted with caution.

The 3-fold difference between targeted systems with IgG and $F(ab')_2$ was a reproducible observation, even though the antibodies were present at the same molarity. As expected, monovalent F(ab') fragments mediated little or no vesicle binding. Excess (0.1 mM) free Tnp-lysine, added to the pre-incubation and incubation, inhibited the interaction almost completely.

Two observations indicated qualitatively that binding of vesicles to the cell surface could not easily be reversed: (i) cells from the targeted IgG system were washed by centrifugation 0, 1, 2 and 3 times with iced medium. Cells examined

TABLE III

FLOW MICROFLUOROMETRIC MEASUREMENTS OF CELL FLUORESCENCE AFTER INCUBATION WITH VESICLES CONTAINING 10 mM 6-CARBOXYFLUORESCEIN (V_T) AND 200 mM 6-CARBOXYFLUORESCEIN (V_F)

V_T was much greater in targeted incubations than in controls, whereas V_F was about the same in all cases. See text for conditions of incubation. Note that vesicle concentrations used in measuring V_F were 35% less than those used for V_T.

Antibody	Tnp on cells	Dnp on vesicles	Soluble inhibitor (Tnp-lysine)	Cell-associated dye (vesicle-equivs./cell)	
				Total (V_T)	Released into cell (V_F)
Targeted					
anti-Tnp	+	+		4 100	370
				4 150	360
				4 510	
$F(ab')_2$	+	+		13 800	n.d. *
				13 500	
Controls					
normal IgG	+	+		140	240
anti-Tnp	—	+		610	260
anti-Tnp	+	—		240	340
anti-Tnp	+	+	+	310	n.d.
F(ab')	+	+		640	n.d.
$F(ab')_2$	—	+		40	n.d.
anti-Tnp	—	+	+	150	n.d.
$F(ab')_2$	+	—		230	n.d.
anti-Tnp	+	—	+	300	n.d.
anti-Tnp	—	—		40	290
					280
—	—	—		n.d.	280

* n.d., not determined.

after each centrifugation showed, respectively, 100, 95, 95 and 91% of the initial post-incubation fluorescence, (ii) after washing, an aliquot of cells was incubated at 5°C for 40 min with 0.1 mM Tnp-lysine; fluorescence decreased only 13%.

For targeted incubations with anti-Tnp at 37, 23 and 3°C, V_T was 4100, 1500 and 550 vesicle-equivalents/cell, respectively. Addition of the metabolic inhibitors sodium azide (0.1%) and 2-deoxy-D-glucose (50 mM), in combination, to the pre-incubation and incubation at 37°C had no effect on binding. These concentrations of inhibitor suffice to block active lymphocyte functions such as capping and cytotoxicity.

(ii) Free intracellular 6-carboxyfluorescein (V_T). The last column in Table III shows the results of flow microfluorometry on cells incubated with 200 mM 6-carboxyfluorescein vesicles. There was little difference between the targeted system and controls in the amount of 6-carboxyfluorescein released from vesicles into the cells (V_F). The average cell fluorescence in the targeted incubations was 370 vesicle-equivalents; in the controls, 290 vesicle-equivalents. Even the slightly greater apparent incorporation in the former could be accounted for by assuming that quenching was only 98% effective or that bound vesicles leaked a small amount of dye into the area of the cell while passing through the laser beam.

The values of V_F in Table III are over an order of magnitude above background. The fluorescence of untreated cells, subtracted from each measurement, was 12 vesicle-equivalents, and exposure to vesicles for about 10 s (followed by the usual washing) did not significantly increase background. It should be emphasized, therefore, that the vesicles used in this study are capable of releasing their contents into both un-modified and Tnp-modified lymphocytes (to the extent that they interact with the cells at all) in the absence of anti-Tnp. The failure to release increased amounts of material into the cells after antibody-mediated binding will be discussed later.

Lymphocytes showed no appreciable fluorescence if incubated with 2.2 μM free dye in the presence of vesicles containing only medium I. From this we conclude that, (i) in incubations with vesicles containing 6-carboxyfluorescein essentially all of the cell-associated fluorophore is derived from the vesicles, and (ii) that adsorption of free 6-carboxyfluorescein molecules to the cell surface is negligible.

Discussion

We conclude from these studies that divalent antibody can bind hapten-modified vesicles to hapten-modified lymphocytes. However, targeted binding led to little or no increase over spontaneous background levels in the amount of vesicle contents released into the cells. As summarized in Table I, these conclusions are based on information from three complementary techniques of observation: fluorescence microscopy, fluorometry and flow microfluorometry. Results from these three were in good agreement with each other.

Antibody-mediated binding

We could not distinguish by fluorescence microscopy whether the vesicles

were bound to the cell surface singly or as aggregates. In any case, they apparently did not cover a large fraction of the cell surface. The largest number of vesicles bound (in the targeted system with $F(ab')_2$) was 14 000. If close-packed on the membrane, this number would taken up approx. 5% of the total area. Failure to dissociate bound vesicles from the cells by repeated washing and by excess free hapten indicates multivalent binding.

$F(ab')_2$ fragments of IgG anti-Tnp mediated three times as much binding of vesicles as did whole antibody. The difference might result from changes in flexibility of the hinge region or relief of steric hindrance associated with the Fc portion of whole antibody, but interpretation remains purely speculative at this point. $F(ab')_2$ fragments may be useful in some targeting functions to forestall interaction with the Fc receptors of phagocytes.

Failure to increase intracellular release of vesicle contents

The vesicles used in these studies are capable of transferring small but definite amounts of their contents into the cytoplasmic compartments of lymphocytes, in the absence of targeting. This is shown by the control incubations (with either dioleoylphosphatidylcholine or dioleoylphosphatidylcholine-Dnp-caproyl phosphatidylethanolamine vesicles), as indicated in Table III. Furthermore, the data summarized in Table I indicate that little, if any, 6-carboxyfluorescein remained in adsorbed vesicles in the controls. These findings were expected, since earlier work from our own laboratory [18,19] and that of Huang and Pagano [17,20] with dioleoylphosphatidylcholine vesicles (in the absence of a targeting moiety) had yielded the same pattern: incorporation of vesicle contents into the cells but no residual adsorbed vesicles. Fusion appeared to be the major mechanism of incorporation (though a metabolically-passive form of adsorptive pinocytosis could not be ruled out conclusively). More recent studies, by fluorescence photobleaching [31], showed most of the incorporated fluorophore to be free to circulate in the cytoplasmic space (unpublished data). Use of fetal calf serum in the present study decreased the non-targeted incorporation but by no means eliminated it. It is natural to ask, then, why the contents of antibody-bound vesicles were not similarly incorporated.

While the molecular details of liposome-cell fusion have not been worked out in any system, recent work in our laboratory [28] on the kinetics with non-targeted vesicles suggests two separable components. One of these is easily saturable, consistent with the notion of a finite number of "sites". The sites are not sensitive to proteolytic agents and it is tempting to speculate that they represent the bare patches of lipid hypothesized to play a role in fusion [32]. Vesicles attached to Tnp groups on surface proteins might not be in the right place for interaction with these sites. It must be observed, however, that the saturable incorporation is effectively blocked by 10% fetal calf serum, as used in the present study, and that it saturates at low levels of uptake in any case. Thus, the sites might not have been expected to play a significant role in incorporation after targeted binding.

Whether bound to trinitrophenyl hapten on the surface proteins or the surface lipids, the vesicles might be located too far from the plasma membrane bilayer to allow fusion. Indeed the targeting antibody molecules themselves might be a barrier to the close approach of the vesicle and cell membrane bilayers. How-

ever, untargeted 6-carboxyfluorescein transfer is not inhibited by coating of the cells or vesicles with antibody (see Table III).

Because of the targeting system used, most of the vesicles might be bound to other vesicles rather than to the cell directly. Since TNBS-modified surface proteins of human lymphocytes do not patch or cap, the patchy appearance of fluorescence on the cells in Fig. 1a may have resulted from formation of clusters of DNP-vesicles not induced by patching of the underlying surface proteins. We saw no evidence of vesicle agglutination by antibody in these studies, but formation of small aggregates cannot be excluded. Use of hybrid antibodies in targeting would reduce interference from vesicle-vesicle and cell-cell interaction.

Implications for targeting

The implications of this work for research and medical applications are clear: the use of antibody to bind hapten-modified vesicles to hapten-modified cells may serve as a mechanism for selective delivery of vesicles and their contents to the surface of a cell, and that may be sufficient for some purposes. But binding does not necessarily lead to intracellular delivery, at least in the non-phagocytic lymphocyte. Lysophosphatidylcholine has been found to enhance fusion in non-targeted systems [33,34]; we have not yet determined whether it will do so as well with antibody-bound vesicles. It also remains to be seen whether the results obtained here with Tnp and anti-Tnp hold generally for other antigen-antibody pairs and other experimental conditions. Recent studies of the binding of Dnp-modified vesicles to cells of the murine myeloma MOPC 315 (which bear on their surface an immunoglobulin A with high affinity for Dnp) give an analogous result: little or no transfer (Leserman et al., unpublished).

In contrast to our work, previously reported studies of antibody-mediated targeting were done with actively endocytotic cells. Gregoriadis and Neerunjun [15] formed large multilamellar liposomes in the presence of IgG specific for surface antigens on each of three tissue culture lines. Antibodies were assumed to associate with liposomes by their Fc portions, and the resulting liposomes showed preferential binding to the corresponding cells. When the cells were fractionated, most of a liposome-entrapped drug was recovered from the lysosomal fraction, consistent with predominance of endocytosis. Weissmann and co-workers [13,14] formed multilamellar liposomes in the presence of heat-aggregated immunoglobulin and stimulated phagocytosis via the Fc receptor. Again, uptake was largely into the lysosomal system. For some purposes, introduction of membrane-impermeant molecules into the cell by phagocytosis is certainly preferable (e.g., for delivery of hydrolytic enzymes to the lysosomes [3,13,14]), but for transfer of material to the cytoplasm it will not always be effective.

In vivo applications of targeted liposomes remain for the future, and several major questions as to their utility remain unanswered: (i) although lysosomal deficiencies may be corrected by targeting via phagocytes' Fc receptors, specific cell surface antigens useful as targets for therapy (e.g., tumor antigens) have not been defined; (ii) immunological sensitization of the host to the targeting moieties (e.g., antibodies) may be a problem; (iii) it remains to be demonstrated that targeted vesicles could be administered in a way (route,

dose, timing) sufficient to achieve effective binding at the relevant in vivo site; (iv) as demonstrated in this study, binding to the cell surface does not ensure transfer to the cell interior.

Appendix

For calculations we assume two populations of cell-associated 6-carboxyfluorescein molecules: N_V molecules per cell remain in vesicles bound to (or intact within) the lymphocyte; N_F molecules are free within the cell. Then F, the observed fluorescence per cell, is

$$F = F_0(\alpha_V N_V + \alpha_F N_F) \quad (1)$$

where F_o is the fluorescence yield of a 6-carboxyfluorescein molecule under standard conditions (i.e., in medium II at 23°C) and each α is the ratio of 6-carboxyfluorescein fluorescence in the specified environment to its fluorescence in the standard state. Eqn. 1 applies to both bulk fluorometry and flow microfluorometry.

If Triton is added to break up cells and vesicles, releasing 6-carboxyfluorescein into the medium (i.e., into the standard state), the fluorescence is

$$F_{\text{post-Triton}} = F_0(N_V + N_F) = F_0 N_T \quad (2)$$

where N_T is the total cell-associated fluorophore. Clearly, equations analogous to Eqns. 1 and 2 apply also to suspensions of vesicles alone.

Finally, we express the results in "vesicle-equivalents", that is in a form normalized by the number of molecules of 6-carboxyfluorescein calculated to be in a vesicle:

vesicle containing 200 mM 6-carboxyfluorescein, $V_i = N_i/260$;
vesicle containing 10 mM 6-carboxyfluorescein, $V_i = N_i/13$.

where i = T, F, or V (3)

The measured fluorescence indicates only the state at the end of incubation. If bound vesicles leat at the same rate as free vesicles in suspension, V_V is about 14% less than the number of vesicles bound. Because of 6-carboxyfluorescein leakage from the lymphocytes during incubation, V_F is less than total incorporation by approx. 30%.

We determined the fluorescence ratios α_V and α_F from measurements on lymphocytes or vesicle suspensions before and after addition of Triton. Lymphocytes incubated with high concentrations of free dye (no vesicles) and then washed yielded $\alpha_F = 0.72 \pm 0.01$ (standard error, five experiments). This value differed from unity at least in part because the intracytoplasmic pH is lower than that of the standard medium; the fluorescence efficiency of 6-carboxyfluorescein drops to low values in acid media, with an apparent pK of about 6.3. There is no a priori assurance that free dye enters the same intracellular compartment(s) as does 6-carboxyfluorescein from vesicles, but identical kinetics of 6-carboxyfluorescein release from the cell in the two cases and indistinguishable appearances in fluorescence microscopy (unpublished data) support the supposition. For vesicles containing 200 mM 6-carboxyfluorescein,

$\alpha_V \approx 0.01$. The exact value is uncertain because of the contribution of small amounts of leaked dye. For vesicles containing 10 mM 6-carboxyfluorescein, $\alpha_V \approx 0.6$ (i.e., approx. 40% self-quenched).

For purposes of calculation we make the approximation that $\alpha_F = \alpha_V$ (10 mM) = 0.7: then, V_T can be determined from flow microfluorometry experiments with vesicles containing 10 mM 6-carboxyfluorescein using an expression derived from Eqns. 1 and 3: $V_T = V_V + V_F = F/(0.7 \cdot 13 \cdot F_0)$. Eqns. 2 and 3 are used to determine V_T from post-Triton fluorometric readings after incubations with vesicles containing 200 mM 6-carboxyfluorescein. With the additional approximation that α_V (200 mM) = 0, we use Eqns. 1 and 3 to calculate V_F from data obtained with vesicles containg 200 mM 6-carboxyfluorescein: $V_F = N_F/260 = F(0.7 \cdot 260 \cdot F_0)$. These approximations are necessary for explicit calculations, but should not be allowed to obscure a fundamental point: the major theses of this paper depend only on the very large difference between α_V (10 mM) and α_F in one hand and α_V (200 mM) on the other. That is, they depend on the nearly 100-fold differences in fluorescence efficiency obtainable from self-quenching.

Acknowledgements

We thank Dr. J. Wunderlich for use of the FACS and C. Neels for preparing the lymphocytes. We are grateful to Drs. P. Dragsten, W. Terry and M. Berman for reading the manuscript. We wish to acknowledge a continuing dialogue with Drs. W. Hagins and S. Yoshikami, contributing to a progressive development of the fluorescence methods.

References

1 Poste, G. and Allison, A.C. (1973) Biochim. Biophys. Acta 300, 421—465
2 Tyrrell, D.A., Heath, T.D., Colley, C.M. and Ryman, B.E. (1976) Biochim. Biophys. Acta 457, 259—302
3 Gregoriadis, G. (1976) N. Engl. J. Med. Part I, 295, 704—10; Part II, 295, 765—770
4 Pagano, R.E. and Weinstein, J.N. (1978) Annu. Rev. Biophys. Bioeng. 7, 435—468
5 McDougall, I.R., Dunnick, J.K., McNamee, M.G. and Kriss, J.P. (1974) Proc. Natl. Acad. Sci. U.S. 71, 3487—3491
6 Batzri, S. and Korn, E.D. (1975) J. Cell Biol. 66, 621—634
7 Jonah, M.M., Cerny, E.A. and Rahman, Y.E. (1975) Biochim. Biophys. Acta 401, 336—348
8 Juliano, R.L. and Stamp, D. (1975) Biochem. Biophys. Res. Commun. 63, 651—658
9 Pagano, R.E. and Huang, L. (1975) J. Cell Biol. 67, 49—60
10 Poste, G. and Papahadjopoulos, D. (1976) Proc. Natl. Acad. Sci. U.S. 73, 1603—1607
11 Pagano, R.E. and Takeichi, M. (1977) J. Cell Biol. 74, 531—546
12 Juliano, R.L. and Stamp, D. (1976) Nature 261, 235—238
13 Weissmann, G., Bloomgarden, D., Kaplan, R., Cohen, C., Hoffstein, S., Collins, T., Gotlieb, A. and Nagle, D. (1975) Proc. Natl. Acad. Sci. U.S. 72, 88—92
14 Cohen, C.M., Weissmann, G., Hoffstein, S., Awasthi, Y.C. and Srivastava, S.K. (1976) Biochemistry 15, 452—460
15 Gregoriadis, G. and Neerunjun, D.E. (1975) Biochem. Biophys. Res. Commun. 65, 537—544
16 Gregoriadis, G., Neerunjun, D.E. and Hunt, R. (1977) Life Sci. 21, 357—369
17 Huang, L., Ozato, K. and Pagano, R.E. (1976) Abstr. Fed. Proc. 35, 1703
18 Weinstein, J.N., Yoshikami, S., Henkart, P., Blumenthal, R. and Hagins, W.A. (1977) Science 195, 489—492
19 Blumenthal, R., Weinstein, J.N., Sharrow, S.O. and Henkart, P.A. (1977) J. Supramol. Struct., Supplement 1, 160
20 Huang, L., Ozato, K. and Pagano, R.E., J. Memb. Biochem., in the press

21 Uemura, K. and Kinsky, S.C. (1972) Biochemistry 11, 4085—4090
22 Weinstein, J.N., Blumenthal, R., Sharrow, S.O. and Henkart, P.A. (1977) Biophys. J. 17, 81a
23 Hauser, H., Oldani, D. and Phillips, M.D. (1973) Biochemistry 12, 4507—4517
24 Lohrman, H.P., Novikovs, L. and Graw, R.G. (1974) J. Exp. Med. 139, 1553—1567
25 Shearer, G.M., Rehn, T.G. and Schmitt-Verhulst, A.-M. (1976) Transplant. Rev. 29, 224—248
26 Shearer, G.M. (1974) Eur. J. Immunol. 4, 527—533
27 Forman, J., Vitetta, E.S. and Hart, D.A. (1977) J. Immunol. 118, 803—808
28 Blumenthal, R., Weinstein, J.N., Sharrow, S.O. and Henkart, P. (1977) Proc. Natl. Acad. Sci. U.S. 74, 5603—5607
29 Loken, M.R. and Herzenberg, L.A. (1975) Ann. N.Y. Acad. Sci. 254, 163—171
30 Julius, M., Sweet, R.G., Fathman, C.G. and Herzenberg, L.A. (1974) Proc. First Los Alamos Life Sci. Symp., Atomic Energy Commission Series (CONS 73-1007), 107
31 Axelrod, D., Koppel, D.E., Schlessinger, J., Elson, E. and Webb, W.W. (1976) Biophys. J. 16, 1055—1069
32 Ahkong, Q.F., Fisher, D., Tampion, W. and Lucy, J.A. (1975) Nature 253, 194—195
33 Martin, F.J. and MacDonald, R.C. (1976) J. Cell Biol. 70, 515—526
34 Weissmann, G., Cohen, C. and Hoffstein, S. (1976) Trans. Assoc. Am. Phys. Vol. 89, pp. 171—183