Endocytosis of liposomes bound to cell surface proteins measured by flow cytofluorometry

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A new technique for the quantification of cellular receptor-mediated endocytosis has been developed based on the analysis by flow cytometry of ligand-bearing liposomes containing the fluorochrome carboxyfluorescein. Carboxyfluorescein encapsulated at high concentrations in protein A-bearing liposomes is self-quenched. Binding and internalization of such liposomes by cells via antibodies directed towards membrane surface determinants results in the release of the liposome-encapsulated carboxyfluorescein into the cytoplasm causing an increase in cell-associated fluorescence. This increase can be quantified on a flow cytofluorometer.

Cell surface molecules such as transport-protein and hormone receptors (recently reviewed by Goldstein *et al.*, 1979; Pearse & Bretscher, 1981; Pastan & Willingham, 1981), immunoglobulin Fc receptors, surface immunoglobulins (Leserman *et al.*, 1980a,b; Lewis *et al.*, 1980; Geiger *et al.*, 1981) and MHC antigens (Leserman *et al.*, 1981; Machy *et al.*, 1982a,b) are endocytosed by cells expressing them. Apart from the first case the importance for normal cell physiology of this endocytosis remains to be properly defined. Most studies of endocytosis have used techniques such as electron or video-intensification microscopy (Goldstein *et al.*, 1979; Pearse & Bretscher, 1981; Pastan & Willingham, 1981), measurements of the uptake of radio-markers coupled to the receptor (Caro & Amatruda, 1980; Dickson *et al.*, 1981; Schwartz *et al.*, 1982) or measurements involving the inhibition of cellular functions resulting from uptake of drugs or toxins coupled to the ligand–receptor complex (Leserman *et al.*, 1981; Machy *et al.*, 1982a,b; Dorland *et al.*, 1981).

We have previously reported that antibody-bearing liposomes containing methotrexate will specifically deliver the drug into cells by a process which is highly suggestive of endocytosis. The extent of drug delivery is dependent on the cell-surface determinant and cell type (Leserman *et al.*, 1981; Machy *et al.*, 1982a,b). Furthermore, small (60–80 nm) but not large (200–400 nm) liposomes delivered drug into non-phagocytic target cells, even

though more drug encapsulated in liposomes became cell-associated in the latter case (Machy & Leserman, 1983). This would be consistent with uptake of liposomes into endocytic vesicles of 150 nm diameter (Pastan & Willingham, 1981).

We report here a technique using cytofluorometry which takes advantage of the self-quenching property of carboxyfluorescein at high concentrations (Weinstein *et al.*, 1977). It involves the use of carboxyfluorescein-containing liposomes to which protein A has been covalently coupled. Protein A-liposomes bind specifically to cells via antibodies directed towards cell membrane surface determinants; the internalization of these determinants and thus of the liposomes results in the release into the cytoplasm and dequenching of encapsulated carboxyfluorescein, causing an increase in the intensity of cell-associated fluorescence. Using this technique we demonstrate the differential endocytosis of two cell membrane proteins, an MHC-encoded antigen and fibronectin. These results are in accord with the earlier observations that liposome encapsulated methotrexate and carboxyfluorescein are bound and internalized by cells as a function of the targeted cell-surface determinant (Leserman *et al.*, 1981; Machy *et al.*, 1982a). This technique permits rapid measurement of endocytosis on single cells.

Experimental

Antibodies and liposomes

H100-30/33 is an IgG2b, kappa, anti-H-2K^k monoclonal antibody (Lemke & Hammerling, 1981). Anti-fibronectin serum (kindly donated by

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; MHC, major histocompatibility complex.

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Dr. M. Hirn, Centre d'Immunologie de Marseille) was obtained by immunizing rabbits against bovine serum fibronectin (IBF, France). Protein A-liposomes were prepared as previously described (Leserman *et al.*, 1980c). Briefly, small, sonicated liposomes composed of 65% (molar) dipalmitoyl phosphatidylcholine, 34% cholesterol and 1% *N*-hydroxysuccinimidyl-3-(2-pyridylthio)propionate-modified phosphatidylethanolamine, containing 40 mM-carboxyfluorescein, were incubated (16 h) with protein A modified with 10 mol of *N*-hydroxysuccinimidyl-3-(2-pyridylthio)propionate per mol of protein A at a final protein A concentration of 200 µg/ml.

Cell culture and incubation and conditions

T cells (Julius *et al.*, 1973) from CBA/J mice (CSEAL-CNRS, Orléans, France) were incubated (30×10^6 cells, 37°C, 7% CO₂) in tissue culture flasks with 20 ml of RPMI 1640 medium (Gibco) supplemented with 5% (v/v) heat-inactivated foetal calf serum (Gibco), 2 mM-glutamine, 5×10^{-5} M-2-mercaptoethanol, penicillin, streptomycin and, in order to induce proliferation, 2 µg of concanavalin A/ml. After 40 h the cells were harvested and resuspended in a supplemented medium without mitogen at a concentration of 8×10^6 cells/ml. L cells (kindly donated by Dr. F. Lemonnier, Centre d'Immunologie de Marseille) cultured in RPMI medium supplemented with 7% (v/v) foetal calf serum were harvested by treatment with 1.5 mM-EDTA in phosphate-buffered saline (5 min, 37°C) to detach the adherent cells, washed and resuspended at the same concentration. For the binding assay, 50 µl aliquots of cells were placed in conical wells of 96-well microtitre plates and 10 µl of H100-30/33 at 40 µg/ml final concentration or anti-fibronectin serum at 1:100 final dilution were added to the appropriate wells. After a 30 min incubation at 4°C, cells were washed once and resuspended in fresh medium. Protein A-liposomes (10 µl) representing 220 pmol of carboxyfluorescein were then added to all sample wells and incubated (4°C) for 90 min. Free protein A (80 µg/ml) was added to the relevant wells a few minutes before the liposomes. The cells were then washed three times in liposome buffer (145 mM-NaCl/10 mM-Hepes, pH 7.4) and the associated fluorescence was measured on an Aminco SPF spectrofluorometer (excitation 488 nm, emission 520 nm) after lysing the pellets with 0.5% Triton X-100. Calculations were based on a 20 mM-carboxyfluorescein standard.

Flow cytometry measurements

T lymphoblasts and L cells were harvested as described above, resuspended in RPMI medium supplemented with foetal calf serum (4×10^7 cells/ml) and incubated (4°C, 60 min) in disposable

plastic test tubes in the presence or absence of antibody. After one wash they were re-incubated (4°C, 90 min) in the presence either of protein A-liposomes (equivalent to 40 µg of phospholipid/10⁶ cells) or of fluorescein isothiocyanate-labelled protein A (20 µg/ml; Pharmacia) and washed twice to remove non-bound material. To examine the kinetics of internalization, cells were incubated in a water bath at 37°C and at various times aliquots were transferred into tubes in an ice bath containing three times the volume of medium. This procedure rapidly cooled the cells and terminated metabolic energy-dependent and enzymic processes. In some experiments, NH₄Cl (10 mM) was added at the start of incubation at 37°C. Samples were then analysed on an EPICS V cell sorter (Coulter Electronics, Hialeah, FL, U.S.A.), using an argon-ion laser model 164 (4 W). The laser excitation was 200 mW at 488 nm with a Schott SAL 530 filter in combination with a cut on glass filter at 530 nm. The photomultiplier setting was 600 V. The flow rate was adjusted to 1000 cells/s. After excluding dead cells by light scattering measurements, 10 000 cells were analysed from each sample for fluorescence intensity. Non-cell-associated carboxyfluorescein did not interfere with the measurements because only a small extracellular volume was illuminated.

Uptake of ¹²⁵I-labelled monoclonal antibody

L cells (10^7 cells/ml) were incubated with ¹²⁵I-labelled H100/30/33 antibody (10^7 c.p.m./ml = 10 µg of protein) for 30 min (4°C) and after removing excess unbound material (two washes) half the cells were treated with protein A-liposomes (4°C, 60 min). Aliquots (200 µl, 10^7 cells/ml) of washed cells were then either maintained at 4°C or transferred to 37°C in the presence or absence of NH₄Cl (15 mM). At the specified time intervals, ¹²⁵I-labelled H100/30/33 uptake was terminated by addition of 600 µl of ice-cold acetate/barbital saline (28 mM-sodium acetate/20 mM-sodium barbital/117 mM-NaCl, pH 3). This procedure rapidly cooled the cells and removed 95% of surface-bound antibody. After 10 min, the cells were centrifuged and washed once. Both the cell pellet and pooled supernatant were then counted on a gamma counter and the remaining cell-associated radioactivity was represented as percentage of total. The specificity of binding of the ¹²⁵I-labelled H100/30/33 was verified as it was competitively inhibited by the same unlabelled antibody.

Results and discussion

In the present report we have used concanavalin A-stimulated T lymphocytes from CBA/J mice and the fibroblastoid L cells. Table 1 shows the binding of protein A-liposomes to T and L cells via a

Table 1. Specific binding of protein A-liposomes to T lymphoblasts and L cells preincubated with anti-H-2K^k (H100-30/33) or anti-fibronectin antibodies

Cell	Antibody	Free protein A	Cell-associated carboxyfluorescein (pmol)
T lymphoblasts	H100-30/33	—	4.23
	H100-30/33	+	0.06
	None	—	0.09
L cells	H100-30/33	—	7.77
	H100-30/33	+	0.07
	Anti-fibronectin	—	1.53
	Anti-fibronectin	+	0.17
	None	—	0.08

previously defined (Lemke & Hammerling, 1981) anti-MHC (H-2) monoclonal antibody, an IgG2b, kappa anti-H-2K^k (H100-30/33), and rabbit anti-fibronectin serum (Raff *et al.*, 1979; Ghandour *et al.*, 1982). Both T lymphocytes and L cells bind protein A-liposomes when preincubated at 4°C in the presence of H100-30/33, but not in its absence. Binding also occurred when L cells were preincubated with anti-fibronectin serum. The binding of protein A-liposomes to both cell types can be inhibited by free protein A. We then followed the time-dependent changes in cell-associated fluorescence on an EPICS V flow cytofluorometer. Fig. 1(a) shows a fluorescence histogram of 10000

Fig. 1. Cytofluorometric measurements of the endocytosis of an MHC antigen H-2K and membrane surface fibronectin using liposome-encapsulated carboxyfluorescein

(a), Fluorescence histogram of L cells that had been stained with H100-30/33 (100 µg/ml) and protein A-liposomes (40mM-carboxyfluorescein) at 4°C or after incubation at 37°C for 2h. The time course of change in fluorescence of T cells that had been similarly treated is shown in (b). Cells were stained either with liposomes containing (■) 100mM- or (▲) 10mM-carboxyfluorescein or (★) with fluorescein isothiocyanate-labelled protein A. Calculations are based on setting the lower fluorescence window such as to exclude 90% of the stained cells at time zero as background. The shift towards higher fluorescence channels with incubation at 37°C is then analysed by determining the product of the number of cells that appear between these set windows and their median fluorescence channel. All values are then normalized by obtaining the ratio to the value at zero time. (c) illustrates the effect of NH₄Cl (■, control; ●, + NH₄Cl) on T lymphoblasts that had been stained with H100-30/33 and protein A-liposomes containing 100mM-carboxyfluorescein. The washing step in one series of samples (○) involves incubating the cells in the presence of NH₄Cl for 2h at 37°C, centrifuging (4°C), resuspending in fresh medium and observing the changes at 37°C. In (d), L cells were stained with H100-30/33 and protein A liposomes (40mM-carboxyfluorescein) and incubated (37°C) in the presence (△) or absence (▲) of NH₄Cl, or with anti-fibronectin serum (●). In all cases non-specific staining with protein A-liposomes (i.e. in the absence of antibody) represented less than 2% of the cell-associated fluorescence.

L cells marked with protein A-liposomes via the anti-H-2K^k monoclonal antibody. Most (98%) of these cells were labelled although most of them appear towards the lower fluorescence channels. The profile did not significantly change over a period of 4 h at 4°C (results not shown). When these cells were transferred to 37°C for 2 h, a shift towards higher channels was observed (Fig. 1a). This phenomenon is a distinct property of highly quenched carboxyfluorescein. In the experiment presented in Fig. 1(b), T cell blasts pretreated with anti-H-2K^k monoclonal antibody were incubated with two preparations of protein A-liposomes containing 100 mM- and 10 mM-carboxyfluorescein, respectively, but which were identical in size, lipid composition and density of bound protein A. The fluorescence windows were set to exclude 90% of the cell-associated fluorescence at 4°C as background and the cells' fluorescence increase was measured when incubated at 37°C. This analysis revealed that liposomes containing 100 mM-carboxyfluorescein caused a large increase in fluorescence which continued to rise even after 4 h (Fig. 1b). Cells incubated with 10 mM-carboxyfluorescein liposomes showed no similar increase. The quenching values for these two types of liposomes were 96% and 74%, respectively. Thus, the 100 mM-carboxyfluorescein liposomes carry the potential to amplify the fluorescence signal up to 25 times the initial value when dequenched, whereas the 10 mM-carboxyfluorescein liposomes can maximally increase the signal only by 3–4 times. These measurements represent values that are an aggregate function of three different parameters: (1) the rate of endocytosis of the cell-surface determinant and thus of the liposomes, (2) the rate at which liposome-encapsulated carboxyfluorescein is released into the cell cytoplasm, and (3) the rate at which free carboxyfluorescein is transported out of the cell. For defined preparations of liposomes the latter two parameters will only depend on the initial concentration of encapsulated carboxyfluorescein. Therefore, although the liposomes would be endocytosed at the same rate, their capacity to increase cell fluorescence will be different. Thus in the limiting case, there will be a concentration of carboxyfluorescein below which (3) will be greater than (2), resulting in no increase in cell fluorescence. Fig. 1(b) also shows that there was no increase in fluorescence when fluorescein isothiocyanate covalently coupled to protein A was substituted for carboxyfluorescein in liposomes.

Using bulk fluorometry the quantity of free carboxyfluorescein in cells can be distinguished from that released into the medium by the addition to the medium of Fab fragments of rabbit anti-fluorescein antibody, which quench (Voss *et al.*, 1976) fluorescence in the medium but not that in the cell, or

in cell-bound liposomes (A. Truneh & D. Aragnol, unpublished work). Such measurements have demonstrated that fluorescence quenching release is a function of the targeted determinant, and is inhibited by cytochalasins that disrupt cytoskeletal organization.

In other systems, internalized ligands contained in endocytic vesicles have been suggested to be processed via low pH compartments to lysosomes where they may be degraded by lysosomal enzymes (Goldstein *et al.*, 1979; Pearse *et al.*, 1981; Pastan & Willingham, 1981; Lewis *et al.*, 1980; Dorland *et al.*, 1981). In a neutral pH environment carboxyfluorescein is a negatively-charged membrane-impermeable molecule. We postulated that, once internalized, carboxyfluorescein is charge-neutralized by protonation in a low pH environment, rendering it membrane-permeable and thus free to diffuse out into the cytoplasm (see also Leserman *et al.*, 1981; Machy *et al.*, 1982a). If so, elevating the lysosomal pH by lysosomotropic agents such as NH₄Cl should retard the dequenching of liposome-encapsulated carboxyfluorescein, thus arresting the increase in cell fluorescence. Fig. 1(c) demonstrates such an effect in T lymphocytes. NH₄Cl nearly abolished the increase over a period of 4 h. This phenomenon is analogous to the inhibition by NH₄Cl of the effect of liposome-encapsulated methotrexate when it is delivered to proliferating cells via MHC antigens (Leserman *et al.*, 1981; Machy *et al.*, 1982a). When NH₄Cl was removed by transferring these cells to a fresh medium after 2 h, there was a rapid increase in their fluorescence which did not show any latency. The rate of increase was faster than that of control cells, which is consistent with the idea that the liposomes have already been delivered to an intracellular compartment whose pH can be rapidly altered by the removal of NH₄Cl (Ohkuma & Poole, 1978). A similar suppression of increase in fluorescence was also demonstrated on L cells (Fig. 1d).

Fluorescence microscopic examination of cells confirmed the above impression. Incubation of cells with targeted liposomes in the cold revealed essentially surface-associated fluorescence. Incubation at 37°C led to diffuse cytoplasmic fluorescence. When this incubation was performed in the presence of 10 mM-NH₄Cl the intracellular carboxyfluorescein was principally confined to perinuclear membrane-bound structures resembling lysosomes (results not shown).

The release of quenched fluorescence depends on the cell surface determinant targeted. A comparison of L cells bearing liposomes via antibodies against H-2K^k and fibronectin revealed that only the H-2K^k determinant and not fibronectin was associated with an increase in cell fluorescence (Fig. 1d). Fibronectin is a structural protein expressed on fibroblast

Fig. 2. *Temperature-dependent decrease in the accessibility to acid washing of surface-bound anti-H-2K antibody* L cells pretreated with ^{125}I -labelled H100-30/33 were incubated (4°C) without (a) or with (b) protein A liposomes for 60 min. After removal of unbound material, aliquots were transferred to 37°C ($\blacktriangle$, $\bullet$) or maintained at 4°C ($\triangle$). NH_4Cl (15 mM) was added where appropriate ($\bullet$). At the times indicated material that was still on the surface was removed by acid washing and the percentage of acid-inaccessible radioactivity was measured as described in the Experimental section.

membrane surfaces and is implicated in cell adhesion and cytoskeletal organization (Hynes & Yamada, 1982). Using a previously described assay for measuring internalization of membrane surface proteins (Leserman *et al.*, 1981; Machy *et al.*, 1982a,b) we also observed that fibronectin is very poor at delivering liposome-encapsulated methotrexate into fibroblasts (P. Machy, unpublished work) thus supporting our contention that endocytosis and quenching-release are causally related events.

We have estimated the amount of antibody bound on the surface of L cells that becomes inaccessible to removal by an acid-washing procedure (Olefsky & Kao, 1982), presumably as a consequence of endocytosis. Fig. 2 demonstrates that the quantity of ^{125}I -labelled anti-H-2K^k antibody that remains cell-associated at 37°C increased as a function of time. In the presence of liposomes, this increase was twice as much as in their absence, the implication being that the multivalent protein A-liposomes (about 15 protein A molecules per liposome; Machy *et al.*, 1982b) may induce the internalization of the antibody, thus of the H-2K^k determinant. After 3 h, about 19% of the antibody was endocytosed. NH_4Cl does not inhibit this process.

Fluorescence techniques have been used to study a large number of cellular functions (for review see Taylor & Wang, 1980). In addition, carboxyfluorescein dequenching measurements have been used as probes of both leakage of liposome contents and their delivery into cells (Weinstein *et al.*, 1977, 1978, 1983; Blumenthal *et al.*, 1977; Leserman *et al.*, 1980a). Here for the first time we have applied

liposome-encapsulated carboxyfluorescein as a marker to follow quantitatively endocytosis of cell-surface determinants. This technique can in principle be applied to quantify endocytosis-associated parameters of any membrane surface molecules, including hormone and carrier-protein receptors for which there is a ligand that can be coupled to liposomes. In addition to the fundamental attribute of signal amplification during endocytosis, the present technique shares with conventional flow cytometry the advantages that (a) the measurements are immediate and precise and can be made on individual cells, (b) interfering dead cells can be excluded from measurements and (c) heterogeneous populations of cells can be studied by using scatter parameters or double-staining techniques to analyse or reject cell subpopulations. These measurements are also non-obtrusive, requiring minimal interference with normal cell physiology and can be made on non-proliferating cells. The technique thus offers the possibility of measurement of receptor-mediated endocytosis for cells obtained by venipuncture from humans. Preliminary results also indicate that the technique is amenable to pharmacological studies of endocytosis, overcoming the limitation associated with bioassays involving measurements of inhibition of cellular functions as a result of endocytosis of a ligand.

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