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Estimation by FACS of the delivery of liposome encapsulated macromolecules into myeloid cells

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Summary

Charged and neutral liposomes were loaded with fluorescein (FITC) labelled albumin. The extent to which the FITC-albumin delivered to non-phagocytic HL-60 cells, zeta potential −9.8 mV, by the process of fusion, was monitored using both a microscope and flow cytometry (FACS). Positively charged liposomes were most effective in their delivery whilst those with a negative charge showed very little transference ability. The delivery of FITC-albumin from positively charged liposomes was found to be heterogeneous with regard to cell size in the absence of PEG, and more homogeneous in the presence of the fusogen.

Introduction

The development of an effective non-toxic method for the delivery of macromolecules into the cytosol of living cells is a necessary prerequisite in the study of control mechanisms of cellular processes and in optimization of drug delivery systems. Since there are difficulties in studying the intracellular behaviour of macromolecules in cell extracts, many laboratories monitor the effects of isolated macromolecules on cells in tissue culture (McCutchan and Pagano, 1968; Bhargava and Shanmugam, 1971; Lane et al., 1971; Graessman et al., 1973). The most efficient method currently employed is the laborious technique of introducing such compounds into the cytoplasm by microinjection. Alternatively, many workers use cell fusion with erythrocyte ghosts as carriers and either Sendai virus (Furusava et al., 1974); Schlegel and Rechsteiner, 1975) or polyethylene glycol (Schlegel and Mercer, 1980) as a fusogen. Unfortunately, proteases present on erythrocyte ghosts, degrade proteins which have been loaded into the cells (Bird et al., 1983). Liposomes not only are free of any of the problems associated with the previous two techniques, but also have the additional advantages of versatility in composition, size, and the ability to encapsulate many different macromolecules. Furthermore, liposomes have been considered as vehicles for introducing membrane-impermeant substances either by membrane fusion or by phagocytosis into cells (Poste and

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Papahadjopoulos, 1976; Pagano and Weinstein, 1978; Finkelstein and Weissman, 1978; Poste, 1980). However, liposomal vector efficiency varies, depending more on the targets than on the liposome type used (Finkelstein and Weissman, 1978; Gregoriadis, 1980). To design more effective liposomal carriers, the various possible mechanisms of interaction between the liposomes and target cells must be analysed for each given set of liposomes and cells. The therapeutic potential of liposomes as drug delivery systems is also dependent on their ability to deliver efficiently the drug to target cells. Significant advances have been made in the ability to direct liposomes to specific target cells but only limited studies have been directed towards generating liposomes capable of releasing entrapped protein (proteoliposomes) in particular biological environments. Apparently, no data are available on the interaction of proteoliposomes with HL-60 cells. We have investigated the necessary requirement for effective cytoplasmic delivery of proteins entrapped in liposomes to HL-60 cells. The effect of the different charge characteristics of the liposomes on the efficiency of delivery was quantified using a combination of microscopical measurements and flow cytometry (FACS). FACS (fluorescent activated cell sorting) allows the rapid analysis of the fluorescence associated with single cells in a population of many millions of cells. Macromolecules which are labelled with a fluorescent probe can thus be delivered to cells and the distribution in the population can rapidly be determined. Additionally, the computer-assisted FACS system allows accurate and rapid measurements of size and density of particles or cells in both homogeneous and heterogeneous populations.

Materials and Methods

Cells and media

The HL-60 cell line is a unique, non-phagocytic leukaemic human cell line, originally isolated from the peripheral blood leukocytes of a female patient with acute promyelocytic leukaemia.

The HL-60 cells were cultured in RPMI 1640 medium (Gibco, Glasgow) containing 10% foetal calf serum, incubated at 37 °C in an atmosphere of 5% CO_2 : 95% air as described previously (Langdon and Hickman, 1987).

Prior to the fusion experiments, microelectrophoretic studies were performed on these cells using a Rank-Particle-Microelectrophoresis apparatus, Mark II (Rank Bros, Cambridge).

Entrapment of FITC-albumin into liposomes

Liposomes for fusion experiments were prepared by the Bangham technique (1965). We used fluorescein isothiocyanate (FITC) labelled albumin (Sigma, Poole, U.K.) as a model protein, the synthetic lipid phosphatidylcholine, and for conferring charge on the liposomes, dicetyl phosphate and stearylamine (all from Lipid Products, Surrey, U.K.). The lipid cholesterol, and charged material, when present, were dissolved in 25 ml chloroform and the solution rotary evaporated at 37 °C. The liposomes were generated from the dry film obtained by addition of 15 ml phosphate-buffered saline (PBS), pH 7.4, containing either 0.01 or 0.1% FITC-albumin for low and high loading of the cells, respectively. This suspension was left to hydrate for 24 h at 4–6 °C. The liposomes were separated by centrifugation at 100 000 $\times g$ for 3 h and redispersed in 5 ml PBS. The entrapment was determined by centrifuging a 5 ml sample, removing the supernatant and sonicating with 5 ml fresh buffer for 15 min in an ice bath.

The fluorescence of FITC-albumin and that of the supernatant were monitored in a spectrofluorimeter (Perkin Elmer LS5) at an excitation wavelength of 490 nm, and emission wavelength of 550 nm. A calibration graph of FITC-albumin in PBS, pH 7.4, was linear over the concentration range studied (3–30 μg/ml).

Cell fusion

The correct medium for the fusion process was determined as follows: 1.92×10^6 HL-60 cells were dispersed in 10 ml of either 0.3% albumin in PBS, PBS, Hanks solution, 40% polyethylene glycol (1500 PEG), or sodium phosphate buffer, pH 7.4. The number of HL-60 cells remaining was monitored every 20 min, for 2 h, by analysing 1-ml samples of the suspension using a Model ZB1

Coulter counter. Cellular damage was determined by the exclusion of a 0.2% solution of trypan blue, using a light microscope. The results were then expressed as the percentage of viable HL-60 cells remaining after each time period.

The fusion procedures were carried out at 37 °C essentially according to the previously described method (Campbell, 1984). A 1 ml aliquot of HL-60 cells (approx. 10^7 cells) in RPMI 1640 containing 10% foetal calf serum (FCS) was added to 1 ml of the liposome suspension in PBS, mixed, and 2 ml of 0.3% albumin in PBS was added dropwise over a period of 30 s. The cells were gently re-suspended with a 2 ml pipette, over a further 30 s, and then left to stand for 30 s. A 5 ml aliquot of 0.3% albumin in PBS (or 40% PEG solution in PBS as fusogen) was then added dropwise over 2 min, followed by the addition of 5 ml of 0.3% albumin in PBS. The suspension was left to stand for 3 min. The suspension was centrifuged at 1000 rpm for 15 min at room temperature, and the supernatant decanted, to remove cells from unbound vesicles.

The pellet was re-dispersed in 5 ml of 0.3% albumin in PBS in order to observe the HL-60 cells which were visualized by the use of an UV light source attached to a Nikon Optiphot microscope. After washing, by dilution, and centrifugation, cells were examined for incorporation of fluorescence and for viability. The percentage of cells taking up FITC-albumin was then determined using a haemocytometer.

The cell solutions were then fixed in readiness for the technique of FACS. The HL-60 cell suspension was centrifuged at 1000 rpm for 5 min at room temperature, to re-form the pellet of cells and the supernatant decanted. The pellet was vortexed in the remaining solvent and 5 ml of 70% ethanol was added dropwise whilst continually vortexing so as to prevent the cells from being fixed as aggregations. The solutions were then wrapped in silver foil, and stored at 4 °C. A Becton Dickinson FACS 440 flow cytometer with an argon laser was used at 490 nm for excitation and 550 nm for emission and data were accumulated and processed on a Consort 40 computer.

The experiments were repeated for positively, negatively, and neutrally charged liposomes.

Results and Discussion

Encapsulation of FITC-albumin into liposomes

The percentage entrapment of FITC-albumin into variously charged liposomes is given in Table 1 for high loadings (0.1% FITC-albumin). The surface charge affected the efficiency of entrapment; the presence of a charged species electrostatically increases the spacing between the phospholipid bilayers, causing an increase in the volume of the aqueous compartment and thus in the amount of drug entrapped. Positively charged liposomes showed maximal entrapment. Encapsulation efficiency was 40.35% for positively charged, 35.32% for neutral and 27.84% for negatively charged liposomes. For low loading (0.01% FITC-albumin) the encapsulated protein, in terms of percentages, was very similar to the high loadings.

Fusion of liposomes with HL-60 cells

The effect of the fusion medium composition on the HL-60 viability is shown in Table 2. As seen 0.3% albumin in PBS allowed optimum maintenance of viability and this medium was used in fusion experiments.

Microelectrophoretic studies showed that HL-60 cells had an overall negative surface charge, zeta potential being -9.8 mV (± 0.68 mV, $n = 8$).

We have sought to achieve the loading of a macromolecular species into HL-60 cells using liposomes of various surface charge. Fusion studies between HL-60 cells and liposomes were car-

TABLE 1

FITC-albumin entrapment by liposomes

Liposome composition	Charge	Entrapment [a] %(±SD)
PC:chol:SA (7:2:1)	positive	40.35 (±0.38)
PC:chol:PA (7:2:1)	negative	27.84 (±0.40)
PC:chol (7:2)	neutral	35.32 (±0.28)

[a] Data are means of five determinations.

PC, phosphatidylcholine; chol, cholesterol; SA, stearylamine; PA, phosphatidic acid.

TABLE 2

Effect of medium on HL-60 cell viability (% of cells remaining after a time, T)

Time (min)	0.3% albumin in PBS	PBS	Hanks solution	40% PEG	Sodium phosphate buffer (pH 7.4)
20	99.8	97.3	99.9	71.3	93.0
40	103.9	97.2	89.7	69.3	83.0
60	95.8	103.6	89.9	67.5	95.2
80	99.0	97.3	99.5	68.9	85.5
100	96.0	90.3	86.4	67.1	89.1
120	94.0	92.1	87.9	66.4	78.3

ried out in both the presence and absence of a fusogen with a liposome : HL-60 cell ratio of approx. 5 : 1. PEG 1500 (40% solution) was chosen as fusogen.

Table 3 lists results of a typical experiment in which FITC-labelled albumin was transferred into human HL-60 cells by fusion with liposomes. The percentage fusion is based in each case on the

TABLE 3

Percentage of HL-60 cells (± SD) fluorescing after the addition of FITC-albumin liposomes, in the presence and absence of PEG

Type of liposomes added to the medium					
Positively charged		Negatively charged		Neutral	
With PEG	Without PEG	With PEG	Without PEG	With PEG	Without PEG
90.32 (±0.53)	89.61 (±0.32)	0.5 (±0.01)	1.19 (±0.25)	87.10 (±0.40)	82.1 (±0.16)

average of two samples in which at least 200 cells were scored. Positively charged liposomes interacted most readily with HL-60 cells: 89.6% (±0.32) of the HL-60 cells took up FITC-albumin from these liposomes. This was probably due to the attraction which allows large areas of liposomal membranes and the cells to adhere, a prerequisite for the fusion process to take place.

The negatively charged liposomes were unable to interact with HL-60 cells. Thus, delivery from

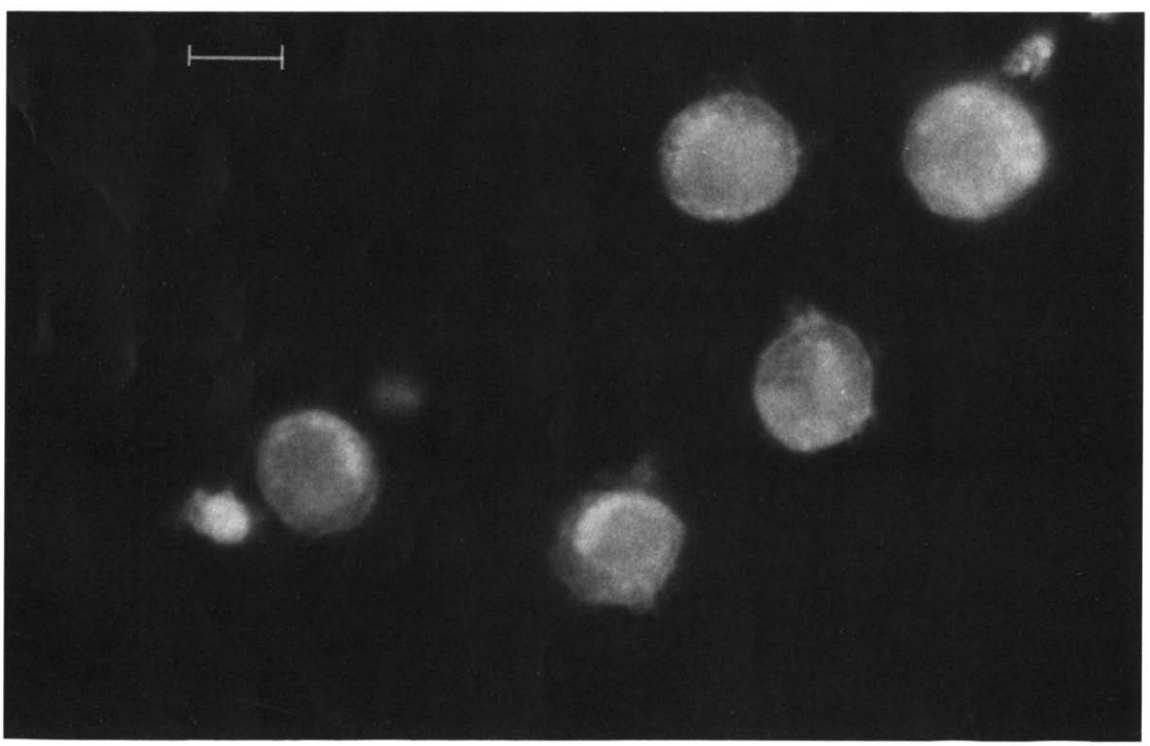

Fig. 1. Photomicrograph of HL-60 cells treated with positively charged liposomes containing FITC-albumin (using ultraviolet light source; scale bar, 12 μm).

these liposomes was limited to a small percentage of HL-60 cells ($1.19 \pm 0.25\%$ fluorescing).

The neutral liposomes delivered FITC-albumin almost to the same extent as positively charged liposomes. Fusion between neutral liposomes and HL-60 cells may be due to hydrophobic interactions rather than charged interactions. Fig. 1 shows photographs of cells treated with FITC-albumin loaded positively charged liposomes. The addition of 40% PEG did not significantly affect the delivery compared to the experiment where it was not used.

Experiments with 'low' loaded liposomes (FITC-albumin starting concentration 0.01% w/v) resulted in poorly fluorescing HL-60 cells. Increasing the initial FITC-albumin concentration to 0.1% w/v resulted in the cells being easily visible which suggests that the concentration of FITC-albumin affects the extent of delivery.

It was found that incubation of HL-60 cells with free FITC-albumin in the presence or absence of empty liposomes did not result in staining of the cells. On the contrary, after a single wash, they appeared as black excluded areas in a slightly fluorescent background (not shown) proving that only liposomes alone were able to deliver the protein (not shown). It may be seen from Fig. 1 that a diffuse cytoplasmic fluorescence is visible, while the nuclear space is clearly excluded. The diffused fluorescence and perinuclear exclusion as seen in Fig. 1 strongly support the notion that fluorescence originates from within the cytoplasm rather than the cell surface, indicating a cytoplasmic delivery of macromolecule. Furthermore, washing the liposome HL-60 mixture several times did not alter the form of staining.

Fluorescent activated cell sorting

The estimation of cell-protein associated fluorescence in individual cells from a total population of 4×10^6 was determined by FACS analysis.

In FACS analysis fluorescently labelled cells are individually sorted and then passed through a beam of monochromatic light from a laser. Data on the number of cells, their sizes as determined by light scatter, granularity and type and intensity of fluorescence emitted by each individual cell were automatically processed. The results are

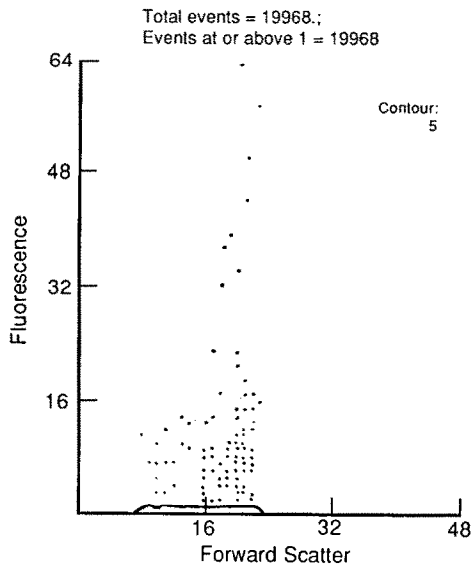

Fig. 2. Flow cytometric dot plot of HL-60 cells.

plotted both as scatter diagrams and as actual relative values.

The results (Figs 3 and 4) agreed with those obtained using fluorescence microscopy. The initial concentration of FITC-albumin affected the extent of delivery to the HL-60 cell (Figs 3a,b and 4a,b). The advantage of FACS is that it measures the size, as right angle scatter (abscissa) and fluorescence of each cell (ordinate) from a population of several million cells.

The FACS data indicated that HL-60 cells did not show significant inherent fluorescence (Fig. 2). Slight fluorescence occurred in the case of cells incubated with FITC-albumin alone (not shown). It is unlikely that FITC-albumin entered the HL-60 cell, since these cells are incapable of phagocytosis (Langdon and Hickman, 1987). It is more probable that a small amount of FITC-albumin was adsorbed onto the cell surface, and despite washings this FITC-albumin could not be removed.

Positively charged liposomes delivered more FITC-albumin, compared to negatively charged liposomes (Figs 3 and 4), reinforcing the theoretical predictions of loading. Unexpectedly, HL-60 cells and negatively charged liposomes do interact, although to a very small extent more likely due to random collisions than attraction, but this was an

insignificant level of delivery. Positively charged liposomes delivered FITC-albumin to a large extent, but only a few of these cells appeared to be capable of taking up large quantities of the protein.

Neutral liposomes showed comparatively less fluorescence transfer than positively charged liposomes (Fig. 5). Under UV light, 82% of HL-60 cells fluoresced (Table 3). It can be concluded that with neutral liposomes, delivery to a large number of cells occurred but the extent of delivery to each cell was poor.

The role of PEG in the fusion process was determined using FACS analysis. It appeared that PEG did not increase the percentage of cells taking up the protein, but did enhance the actual quantity of FITC-albumin that was delivered to each individual cell (Figs 3c, 4c and 5b). In this way, each fusion experiment, irrespective of liposome charge, displayed a greater concentration of

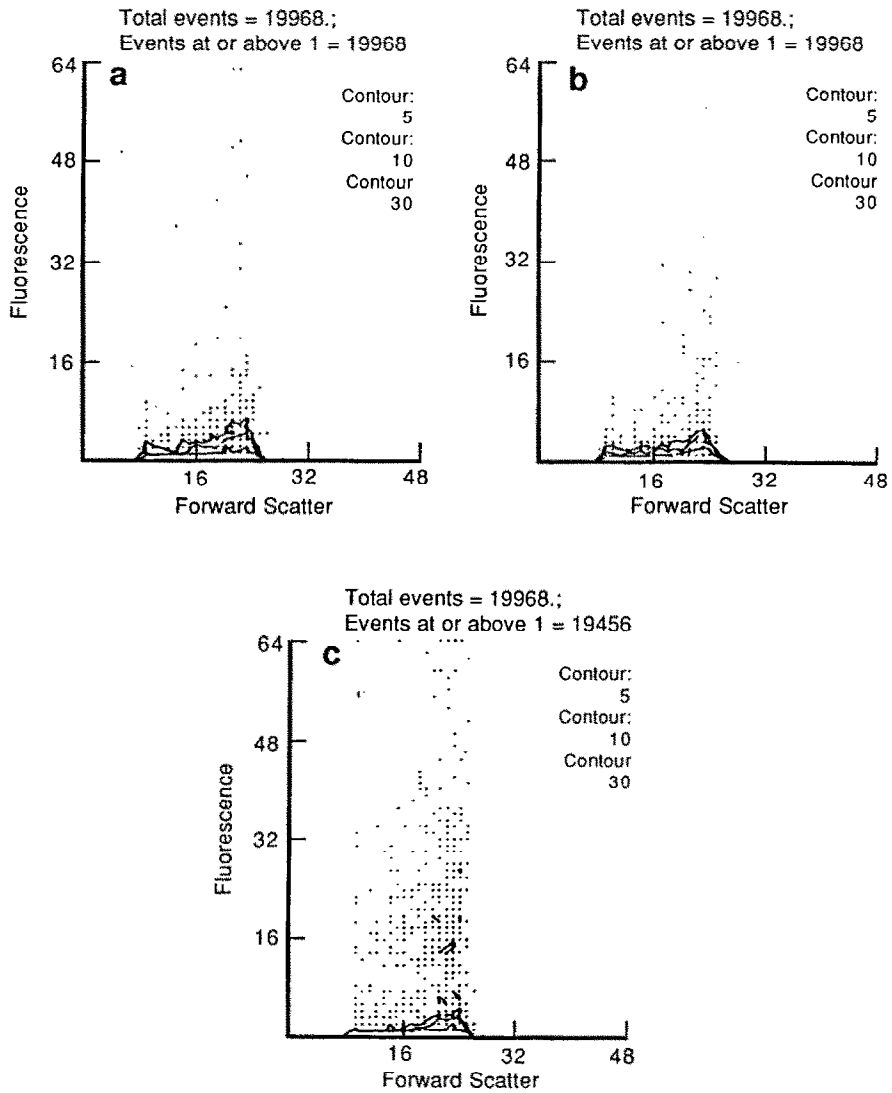

Fig. 3. Flow cytometric dot plots showing the interaction of negatively charged liposomes containing FITC-albumin with HL-60 cells. (a) Low loading, (b) high loading, (c) high loading, PEG treated.

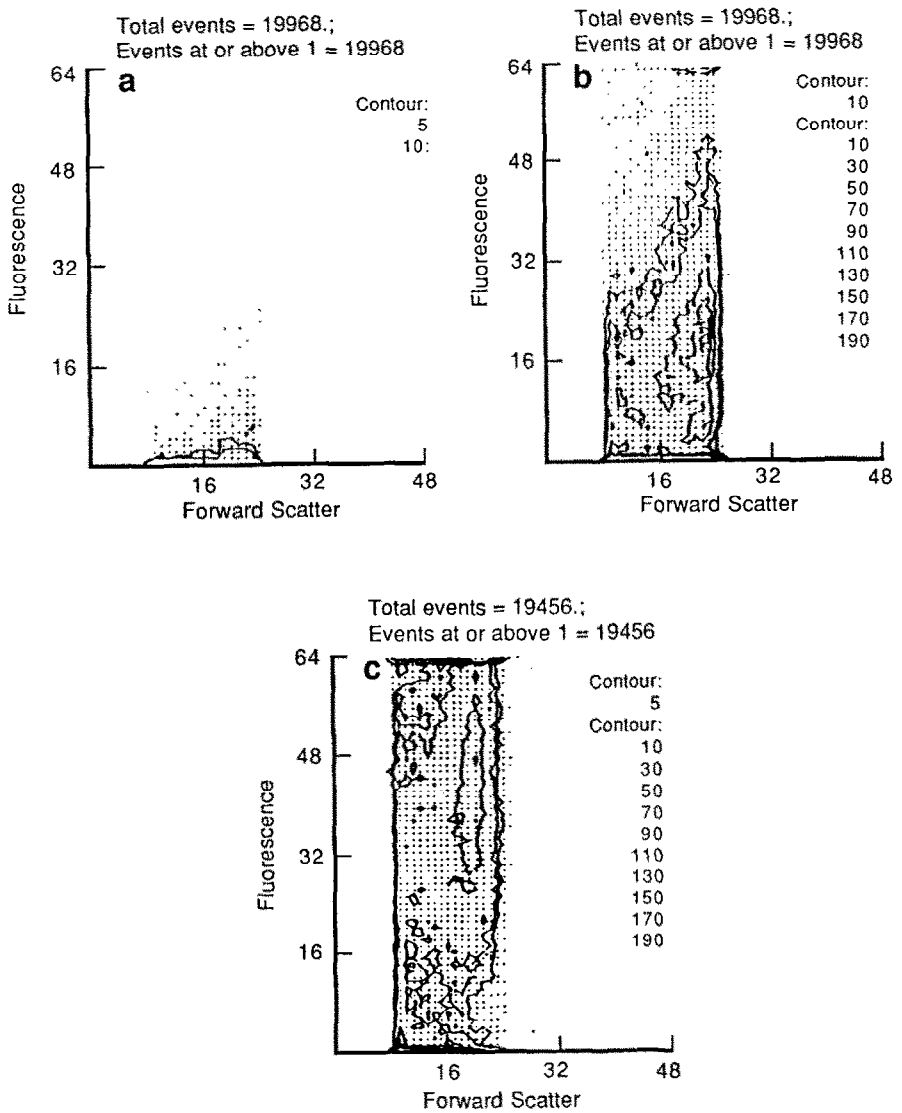

Fig. 4. Flow cytometric dot plots showing the interaction of positively charged liposomes containing FITC-albumin with HL-60 cells. (a) Low loading, (b) high loading, (c) high loading, PEG treated.

fluorescence in the presence of PEG than in its absence.

The data clearly show that negatively charged liposomes were unable to deliver fluorescence-labelled albumin to HL-60 cells. When positively charged liposomes were used, in the absence of PEG, cell-associated fluorescence was distinctively increased in the larger cells of the population. Large cells are approaching the G_2/M phase of the cell cycle and it would be interesting to determine their zeta potential compared to that in G and S phase. When PEG was used, cells of all sizes took up the fluorescently labelled albumin when delivered in positively charged liposomes, and an almost equal distribution was then found in the whole population.

Conclusions

The rapid FACS assay system has enabled us to evaluate, in single cells, the efficiency of charged

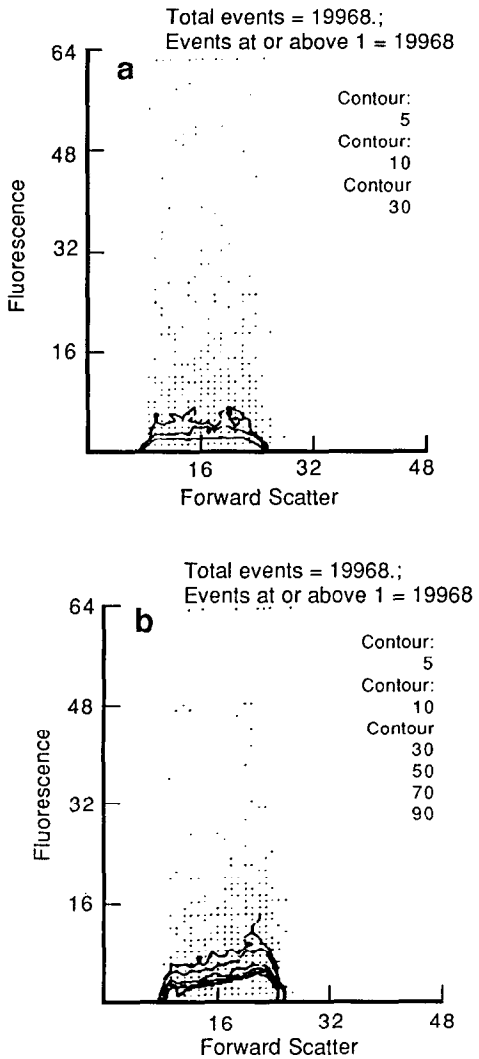

Fig. 5. Flow cytometric dot plots showing the interaction of neutral liposomes with HL-60 cells (high loading). (a) Without fusogen (PEG), (b) with fusogen.

and uncharged liposomes as macromolecule delivery vehicles. We have shown that negatively charged liposomes were unable to deliver labelled albumin to HL-60 cells. Positively charged liposomes, in the absence of PEG, have been demonstrated to be effective in the delivery of biologically active molecules to the larger functionally intact HL-60 cells. In the presence of PEG enhanced drug transfer apparently occurs to the small through to large HL-60 cells.

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