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Analysis and isolation of endocytic vesicles by flow cytometry and sorting: Demonstration of three kinetically distinct compartments involved in fluid-phase endocytosis

(endosome/lysosome/cathepsin B/fluorogenic substrates/fluorescein isothiocyanate-dextran)

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ABSTRACT **The existence of three distinct classes of endocytic vesicles that are part of the pathway of fluid-phase endocytosis has been demonstrated by flow cytometry. Amounts of fluorescent and scattered light were measured on a particle-by-particle basis for unfractionated whole cell lysates from cells incubated with fluorescein isothiocyanate-dextran. After 20 min two different fluorescent populations were observed, and after a 180-min incubation a third highly fluorescent population was found. Since the fluorescein isothiocyanate-dextran per fluid-phase vesicle should be a function solely of the external fluorescein isothiocyanate-dextran concentration, the existence of endocytic compartments with widely different amounts of fluorescence could result from a wide range of sizes of initial endocytic vesicles. However, the kinetics of appearance of the intermediate and highly fluorescent vesicles suggest that these compartments become labeled through fusion with the smaller primary endocytic vesicles.**

The mechanism and role of differential endocytic processing has been approached recently by a number of methods (for reviews, see ref. 1–4). The existence of intermediate vesicles that participate in the endocytic process has been substantiated by a number of laboratories (e.g., refs. 5–11). Many of the details of these pathways are not yet known due to the limitations of the methods used. For example, morphological studies by electron microscopy have the advantage that spatial and structural information may be obtained. However, the method is inherently static, sample sizes are ordinarily limited, and it is difficult to quantitate enzymatic and biochemical properties. Density gradient centrifugation has been very useful due to the possibility of performing biochemical analyses on the resulting fractions. However, the length of time required for fractionation introduces the possibility of changes in vesicle properties, as might be expected from proteolysis or loss of ion gradients. In addition, bulk fractionation techniques are unable to address questions that require particle-by-particle analysis, such as, whether different endocytic probes or different enzymes are contained in the same compartment.

While the usefulness of flow cytometry for the study of endocytosis has been demonstrated (12–16), it has, heretofore, been limited to measurements on whole cells. Flow-microfluorometric analysis of synthetic phosphatidylcholine/cholesterol vesicles has been described by Allen *et al.* (17). The results described in this paper demonstrate the feasibility of using flow cytometry and sorting to characterize unfractionated cell lysates after labeling with endocytic and enzymatic probes. This technique offers the advantages of rapid measurement of large numbers of individual vesicles, the ability to simultaneously measure more than one vesicle property, and the possibility of sorting individual vesicles for further analysis. Preliminary accounts of this work have been presented at meetings.*

MATERIALS AND METHODS

Reagents and Cell Culture. Fluorescein isothiocyanate (FITC)-conjugated dextran (average molecular weight 70,000) was obtained from Sigma. FITC-conjugated microspheres were obtained from Becton Dickinson Immunocytometry Systems.

Swiss albino 3T3 fibroblasts (ATCC CCL92) were obtained from American Type Culture Collection and maintained as subconfluent monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (GIBCO), 100 units of penicillin/ml, 100 μg of streptomycin sulfate/ml, and 1% L-glutamine.

Labeling Conditions. For labeling of compartments involved in fluid-phase endocytosis, cell monolayers were washed with 2 ml of Dulbecco's phosphate-buffered saline (PBS, pH 7.4) and then incubated with various concentrations of FITC-dextran for various times at 37°C. Monolayers were washed eight times with DMEM, once with PBS, and homogenized as described below.

For labeling of vesicles containing proteolytic activity, the assay of Dolbeare and Vanderlaan (18) was used. Monolayers were washed twice with PBS and then incubated in 1 ml of staining solution containing 1 mM benzyloxycarbonyl-alanylarginyl-arginine 4-methoxy-2-naphthylamide, 1 mM 5-nitrosalicylaldehyde, 0.25 mM dithiothreitol, 2 mM EDTA, 0.2 M NH_4OAc (pH 6.0). After incubation at 37°C for 30 min, the cells were washed twice with 0.2 M NH_4OAc (pH 6.0) and then twice with PBS. Homogenates were then prepared as described below. Control plates received staining solution without benzyloxycarbonylalanylarginylarginine 4-methoxy-2-naphthylamide. The specificity of the proteolytic activity was assayed by the addition of leupeptin (100 μg/ml) before staining. Under the conditions used, more than 99% of the hydrolysis of substrate was prevented by this addition.

Preparation of Cell Homogenates. After the washes described above, 1 ml of ice-cold 0.25 M sucrose, 2 mM EDTA, 10 mM Hepes, pH 7.4 was added. All further manipulations were at 4°C. The cells were scraped from the dish with a rubber policeman, and the cell suspension was homogenized with two strokes in a glass-teflon homogenizer. The sample was immediately placed on the FACS 440 for analysis.

Flow Cytometry and Sorting. All analysis and sorting was performed on a FACS 440 equipped with a Consort 40 Data

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Management and Control System (Becton Dickinson Immunocytometry Systems). The 488-nm wavelength line of an argon ion laser was used for excitation. Linear and logarithmic fluorescence (collected using a 530-nm bandpass filter with a 30-nm bandwidth) as well as linear forward scattered light and logarithmic-90°-(side)-scattered light were collected in list mode using 256 channel resolution. A triggering threshold was normally set on forward scatter just above the level resulting from scattering from the sample stream (sheath fluid). Dual parameter histograms were created from the list mode data at 64 × 64 channel resolution. For all experiments, FITC-conjugated microspheres were used for calibration.

RESULTS

Sequential Labeling of Three Endocytic Compartments. Using the fluid-phase endocytic marker, FITC-dextran, three kinetically distinct vesicle classes were observed (Fig. 1). This data was acquired using logarithmic amplifiers to allow simultaneous display of the wide range of scatter and fluorescence values observed. Four regions are shown, corresponding to three populations of subcellular organelles (weak, intermediate, and bright) and one of unlysed cells. The position of the intact cells was determined in a parallel experiment (data not shown). A 20-min incubation with FITC-dextran resulted in two distinct labeled populations. Merion *et al.* (19) have demonstrated a similar resolution of at least two endocytic compartments after a 15-min incubation with FITC-dextran using Percoll gradients. Table 1 shows the mean fluorescence values and the percentages of total particles in the various vesicle compartments. As incubation time increases, the percentage of the weakly fluorescent vesicles decreases, and a highly fluorescent population appears. The kinetics of appearance of this population suggest that it may consist of secondary lysosomes and multivesicular bodies. Note that the intermediate vesicles are 6–8 times as fluorescent as the dim vesicles and that the bright vesicles are 10–13 times more fluorescent than the intermediate vesicles.

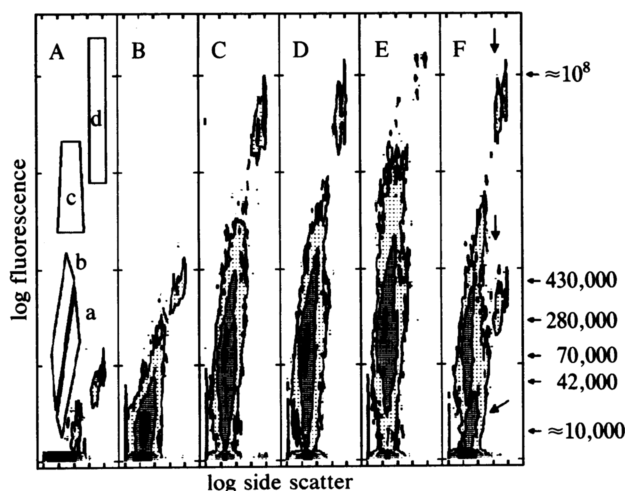

FIG. 1. Detection of endocytic vesicles in cell lysates using flow cytometry. Results of flow cytometric analysis of crude cell lysates are shown for control cells that did not receive any FITC-dextran (*A*) and for cells which were incubated with 50 mg of FITC-dextran/ml for 5 sec (*B*), 20 min (*C*), 60 min (*D*), and 180 min (*E*). Each panel represents 50,000 events. The polygons in *A* show the regions in *C–E* where vesicles which show weak (a), intermediate (b), and bright (c) fluorescence are found. The position of intact fluorescent cells (d) is also shown. The arrows show the average fluorescence of four types of calibration microspheres and the extrapolated positions corresponding to 10,000 and 100,000,000 molecules of fluorescein per event. A mixture of equal volumes of the samples in *A* and *D* was also analyzed (*F*). Note the presence of a population of unlabeled vesicles and two populations of intact cells in this mixture (large arrows). Contours are drawn at 3, 20, and 80 events per bin. Tick marks are placed at approximately one log intervals.

Table 1. Quantitation of vesicle subpopulations

	Vesicle subpopulation					
	Region 1		Region 2		Region 3	
Incubation time, min	Mean fluorescence	% of total	Mean fluorescence	% of total	Mean fluorescence	% of total
20	17	46	108	46	1390	1
60	17	34	120	53	1190	1
180	24	32	212	51	2110	9

Data from Fig. 1. Mean values of fluorescence are in arbitrary units.

To rule out the possibility that the fluorescence associated with these organelles was due to free FITC-dextran, the homogenates from unlabeled cells and those labeled for 60 min were mixed in various proportions and then analyzed as in Fig. 1. If association of probe was taking place, then two changes should be observed after mixing. First, the fluorescence of the labeled populations should decrease, and second, unlabeled vesicles should appear fluorescent. Fig. 1*F* shows that the same labeled populations are present in these mixtures but, more importantly, that an unlabeled population (those at and below the arrow at 10,000 fluoresceins) is also present in an amount proportional to the fraction of unlabeled homogenate present.

Acidification. When isolated by gradient centrifugation, vesicles that are normally acidic, such as lysosomes, are often observed to have dissipated their pH gradient. To investigate whether rapid flow cytometric analysis might enable detection of FITC-dextran-fluorescence quenching due to acidic pH, lysates were prepared from cells labeled for 5–30 min. Fig. 2 shows linear fluorescence versus logarithmic side scattering histograms for these samples. Linear fluorescence was used to allow accurate quantitation of small fluorescent signals. As early as 5 min after addition of FITC-dextran, labeled vesicles can be identified. The average fluorescence of this population is two to three times higher than that from cells labeled for 10 min, indicating that quenching due to acidification has occurred during the

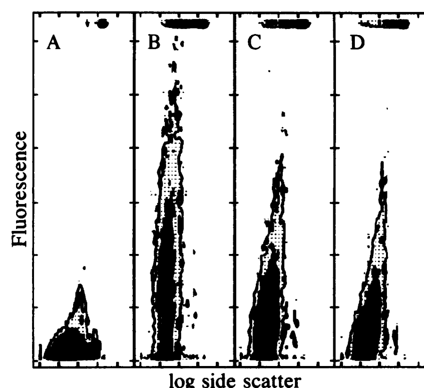

FIG. 2. Kinetics of appearance of endocytic vesicles containing FITC-dextran and quenching due to vesicle acidification. Cells were incubated without (*A*) and with 4 mg/ml FITC-dextran for 5 min (*B*), 10 min (*C*), or 20 min (*D*) and then homogenized. Flow cytometric analysis was performed as for Fig. 1 with the exception that linear fluorescence was recorded to detect differences in fluorescence intensity due to acidification. Each panel represents 50,000 events. Contours are drawn at 5, 20, and 80 events per bin. Tick marks on the abscissa are placed at approximately one log intervals.

second 5 min period. That the vesicles had maintained their pH gradient during the brief period between cell lysis and analysis was verified by comparing the average fluorescence in the presence and absence of a weak base to neutralize acidic vesicles. The fluorescence of vesicles from cells labeled for 15–30 min increased an average of 2.7-fold after the addition of 100 mM methylamine. This increase corresponds to an average vesicular pH of 5.

Cytochemical Characterization of Vesicles. One of the potentially useful applications of the technique described above is the determination of enzyme contents for individual vesicles containing endocytosed probes. To test the feasibility of this approach, lysates from cells incubated with a fluorogenic substrate for the lysosomal enzyme cathepsin B were analyzed by flow cytometry. As described by Dolbeare and Vanderlaan (18) this assay relies on the formation of a Schiff's base between nitrosalicylaldehyde and the liberated fluorophore, 4-methoxy-2-naphthylamine (20). In addition to making the product insoluble and, hence, unable to diffuse from the compartment in which it had been produced, this procedure also shifts the excitation wavelength of the probe into the visible range so that it can be monitored by using 488-nm excitation.

Fig. 3 shows fluorescence versus scatter histograms for homogenates from cells labeled using this procedure. The control sample, which did not receive the substrate, shows some fluorescence due to the nitrosalicylaldehyde (Fig. 3*A*). A similar weakly fluorescent population is also observed in the sample receiving substrate (Fig. 3*B*), but in addition, highly fluorescent vesicles and intact cells are observed. That the staining is specific for proteases is demonstrated by Fig. 3*C*, which shows the results for a lysate from cells that also received 100 μg of leupeptin/ml, an inhibitor of cathepsin B. To substantiate the intravesicular location of the fluorescent product, equal volumes of the samples in Fig. 3 *B* and *C* were mixed and then analyzed (Fig. 3*D*). As discussed above for the FITC-dextran-containing samples, the lack of effect of this mixing on the fluorescence intensity of the labeled population rules out the possibility of artifactual fluorescence due to free probe. In addition, the presence of the leupeptin in the lysate shown in Fig. 3*D* does not affect the fluorescence of the vesicles isolated from the leupeptin-lacking cells, as expected.

Flow Sorting of Specific Populations. Laser-based flow cytometers such as the FACS 440 usually have the capability to sort individual particles based on their measured properties. To test whether the highly fluorescent, FITC-dextran-containing vesicles described above could be isolated by this technique, lysates were prepared from cells labeled for 90 min. This lysate was analyzed as above but with the sorting head drive turned on. The resulting fluorescence versus scatter histogram (Fig. 4*A*) shows some loss of resolution due to interference from stream oscillations induced by the head drive, but the normal range of particle fluorescence is seen. Two regions of this histogram were selected and particles falling into these regions were sorted into separate tubes. Reanalysis of the sorted fractions (Fig. 4 *B* and *C*) confirms that these populations represent distinct compartments and demonstrates the feasibility of rapid separation of homogeneous populations without the use of centrifugation. Microscopic examination of the sorted populations reveals that they are primarily single vesicles (data not shown).

DISCUSSION

The present study demonstrates that direct organelle analysis by flow cytometry provides an effective means for examining questions regarding the processing of endocytosed ligands. The results demonstrate that (*i*) FITC-dextran is internalized into at least three different endocytic compartments, (*ii*) the acidic pH of these compartments is maintained for at least a few minutes after cell lysis, and (*iii*) these compartments can be isolated by sorting for further analysis.

FITC-dextran has been shown by a number of laboratories to be a convenient marker for the process of fluid-phase endocytosis. The amount of FITC-dextran contained in a

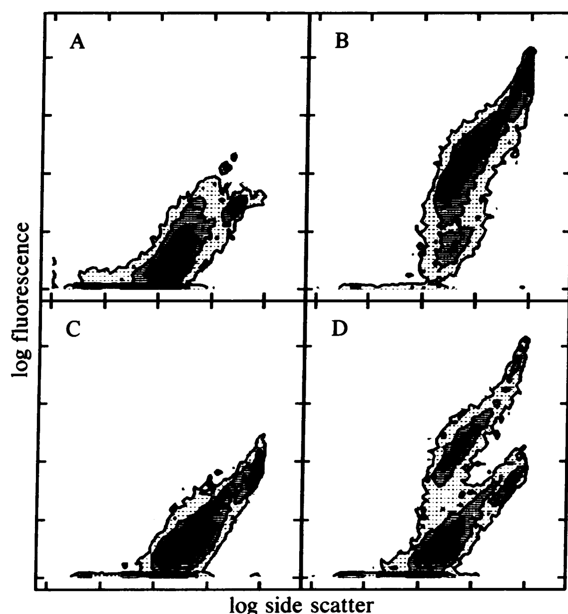

FIG. 3. Detection of vesicles containing cathepsin B activity. Flow cytometric analysis for lysates from cells incubated with (*B* and *C*) and without (*A*) fluorogenic substrate and with (*C*) or without (*A* and *B*) 100 μg of leupeptin/ml. A mixture of equal volumes of the samples shown in *B* and *C* was also analyzed (*D*). Contours are drawn at 5, 20, 40, and 80 events per bin. Tick marks are placed at approximately one log intervals.

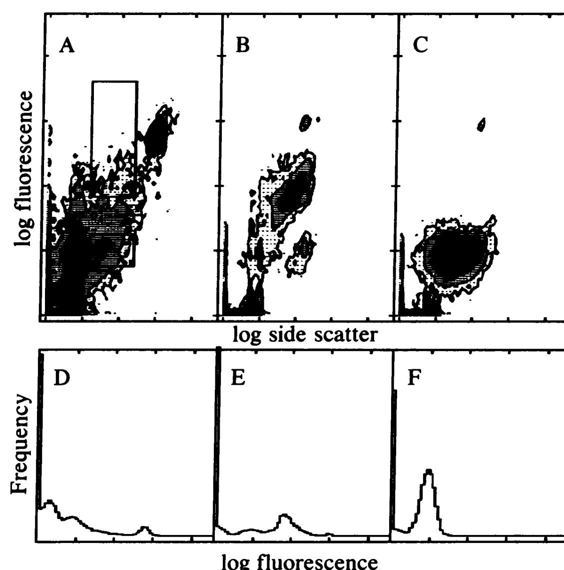

FIG. 4. Isolation of subpopulations of endocytic vesicles by flow sorting. Cells were incubated with 4 mg of FITC-dextran/ml for 90 min and then homogenized. Dual parameter histograms are shown for this whole cell lysate (*A*) and for samples sorted from the upper (*B*) and lower (*C*) regions shown in *A*. Contours are drawn at 2, 6, 20, and 60 events per bin in *A* and at 6, 20, and 60 events per bin in *B* and *C*. Tick marks on the abscissa are placed at approximately one log intervals. Log fluorescence histograms for these samples are also shown (*D–F*).

primary endocytic vesicle should be proportional only to the volume of that vesicle. Thus, the amount of fluorescein fluorescence per vesicle can be used as an indication of the heterogeneity in size of these initial vesicles. The results presented in Fig. 1 indicate that there is some variation in fluorescence per vesicle at early times. However, since a new population is observed on longer incubation (and the relative frequency of the two early populations changes with time), fusion of primary endocytic vesicles with each other, either directly or indirectly, is indicated. Of course, vesicle acidification will induce heterogeneity in fluorescence per vesicle, but these differences are limited to a factor of 5 (compared to the differences of more than 60-fold between the dim and bright vesicles seen in Fig. 1 and Table 1).

A number of intermediate structures, or endosomes, that participate in endocytosis have been identified by electron microscopy (for review see ref. 3). Previous experiments have not addressed the mechanism by which soluble endocytosed material passes through these compartments. For example, it is possible that an incoming primary endocytic vesicle increases its volume by the addition of water to form an endosome (which may undergo a number of structural transformations) and then fuses with one or more primary lysosomes to form a secondary lysosome. However, the results presented here indicate that at least some primary endocytic vesicles must fuse either directly with each other or indirectly by fusion with some other compartment. In addition, since the highly fluorescent compartment seen at 180 min appears to have a size (as judged by light scattering) similar to that of the early compartments, decreases in volume through loss of water and/or membrane may occur during or after this fusion.

The results in Fig. 3 indicate that many of the organelles that are detected by our technique have proteolytic activity. This is in agreement with the results of Storrie *et al.* (8), who found that fluid-phase markers reached an acid phosphatase-containing compartment within 13–15 min, and those of Diment and Stahl (21), who have demonstrated that endocytic vesicles from macrophages acquire cathepsin D activity within minutes after formation and before fusion with lysosomes containing other enzymes. Thus, the distinction between endosomes and lysosomes becomes less clear. Since Golgi-derived vesicles contain proton pumps that can be activated *in vitro* (22), it is clear that additional information on the biochemical contents of compartments involved in endocytosis is needed.

It should be possible to address other questions regarding endocytic processing by using mixtures of fluorescent probes whose fluorescence can be resolved by appropriate combinations of laser lines and optical filters. As the number of parameters that are measured is increased, analysis of the resulting data becomes increasingly difficult. An approach that has proven useful for resolving other types of complex multiparameter flow cytometric data is the use of cluster analysis to resolve subpopulations within a heterogeneous sample (23). These subpopulations can then be identified by using additional probes, by functional studies, or by flow sorting.

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