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ISLET CELL ANALYSIS AND PURIFICATION BY LIGHT SCATTER AND AUTOFLUORESCENCE.

Marnix VAN DE WINKEL, Eddie MAES, Daniël PIPELEERS

Dept. of Metabolism and Endocrinology - Vrije Universiteit Brussel
Laarbeeklaan 103 - B-1090 Brussels, Belgium

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Rat pancreatic A- and B-cells differ in light scatter and flavin-adenine-dinucleotide (FAD)-related fluorescence and are thus represented by two easily distinguishable populations in a fluorescence-activated cell sorter (FACS). Sorting of dissociated islet cells yields highly purified single A- and B-cell preparations. FACS-analysis of islet cells also indicated that FAD-fluorescence in B-cells is reduced within a 5 minute exposure to 20 mM glucose, whereas no variations were observed in A-cell fluorescence nor with 3-O-methylglucose or fructose. FACS-analysis of blood cells and of dissociated liver, parotid, pituitary and pancreatic exocrine cells demonstrated a wide variation in the respective FAD-fluorescence intensities, which could be used for their purification as viable single cells as well as in studying their metabolic redox state.

INTRODUCTION

Glucose homeostasis is regulated by the opposing effects of glucose upon insulin and glucagon release (1). The mechanisms through which glucose inversely regulates the secretory activities of neighbouring cells in the pancreatic islet are however poorly understood. One approach to this question consists in analysing the stimulus-secretion coupling of insulin-containing B-cells separately from that of glucagon-containing A-cells. We recently reported that differences in cell size allow the purification of single B-cells by counterflow centrifugation (2); this method initiated investigations on the glucose-induced response of single B-cells (3) but did not permit analogous studies on A-cells, since the A-cell enriched fraction was still contaminated by 25% B-cells (2). The relatively high flavin content in pancreatic islets (4,5) prompted us to assess whether a fluorescence-activated cell sorter

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(FACS) discriminates better between small and large islet cells through simultaneous measurements of light scatter and endogenous fluorescence intensity due to FAD. This technique allowed a rapid and reproducible purification of single and viable A- and B-cells; we therefore tested its potential use for the preparation of other cell types and for investigations on intracellular redox states.

METHODS

Cell preparations.

Cell suspensions were prepared from various tissues of adult male Sprague Dawley rats (200-250 g). Pancreases were digested with collagenase and separated into an endocrine and exocrine fraction (6,7). Isolated islets were dissociated and eventually purified into an A-cell enriched mixture and into single B-cells (2). Trypsinization (100 µg/ml trypsin; Boehringer) of the exocrine fraction in a calcium-free medium, containing 4 µg/ml bovine pancreatic deoxyribonuclease (1000 U/mg - Boehringer) yielded 30% single cell suspensions. Single liver cells were obtained by dispersion of tissue fragments in calcium-free medium. The preparation of pituitary and parotid cells was similar to that for pancreatic exocrine cells. All cell samples were examined in Hepes-buffered Krebs-Ringer (pH 7.4) containing 2.8 mM glucose and 1% bovine serum albumin (BSA-fraction V - Sigma) (2).

Cell sorting.

The cells were analysed in a fluorescence-activated cell sorter (FACS IV, Becton Dickinson) (8). Light scatter was measured as near forward scattered light intensity(low angle 3°-13°); cellular fluorescence was excited at 488 nm by a 200 mW laser beam (Argon 164-06, Spectra Physics) and its emission measured above 520 nm (LP 520 interference filter, Ditric Optics). Glutaraldehyde-fixed chicken erythrocytes were used for calibration and reference for light scatter and fluorescence intensities. FACS-analysis resulted in a dot display of 2000 particles, plotted according to their relative values in light scatter (abcissa) and fluorescence (ordinate). Particle populations were recognized on the dot plots through a high density of dots with similar scatter and similar fluorescence intensity. These populations were sorted and examined in phase contrast, in electron microscopy and by vital staining with neutral red. The reported results only pertain to populations of single and viable cells.

Characterization of cellular fluorescence.

Islet cell fluorescence was measured by flow cytometry after reduction with sodium dithionite (5 mM), after blocking the respiratory chain with potassium cyanide (0.5 mM), or after photoreduction (9) through a 30 min illumination (Schott KL 1500/325-720 nm, 10 Mlx). FACS-analysis was also performed to evaluate islet cell fluorescence after 5 min exposure to 20 mM glucose, 3-0-methylglucose, or fructose, or following 16 h culture in CMRL-1066 (Gibco, Glasgow). Fluorescence excitation and emission spectra were determined in extracts of single hepatocytes and single B-cells, which had been isolated under the described sorting conditions; the spectra were recorded in a spectrofluorimeter (Jobin-Yvon 3 C, Sobricom) and were compared to those obtained with flavin mononucleotide (FMN) (5).

RESULTS

Sorting of islet cells.

Islet cell preparations enriched in either A- or B-cells yielded different displays on the FACS-dot plot and were each characterized by a distinct single cell population containing 80 to 85% A- and more than 95% B-cells respectively, with a cross contamination of less than 3% (Fig 1a, 1b). When unpurified islet cells were analysed under identical conditions, the same two populations were immediately detected (Fig. 1d) and their sorting resulted in the same degree of islet cell purification. As dead islet cells displayed a lower light scatter and fluorescence, appropriate window setting succeeded in the purification of more than 95% viable cells, as judged by neutral red staining and electron microscopy.

Although dot plots indicate that A-cells scatter less light than B-cells, the scatter histograms of unpurified islet cells correspond to one broad asymmetric peak (Fig. 1e). On the other hand, the difference in autofluorescence of A- and B-cells was much more pronounced (Fig. 1c) and led to two distinct peaks in the fluorescence histograms of unpurified preparations (Fig. 1e).

Sorting of cell preparations from other tissues.

When other rat cell suspensions were examined under identical conditions, each of them also yielded one or more clusters on the FACS-dot-plot, indicating the existence of particle populations with similar scatter and similar autofluorescence intensities; after identifying these clusters as intact single cell populations, their respective scatter and fluorescence intensities were measured and expressed relative to the red blood cell standard (Table 1). The pancreatic exocrine cell fraction was thus found to consist of two viable single cell populations differing in fluorescence intensity. The cellular fluorescence intensity was highest for isolated hepatocytes and lowest for erythrocytes and lymphocytes;

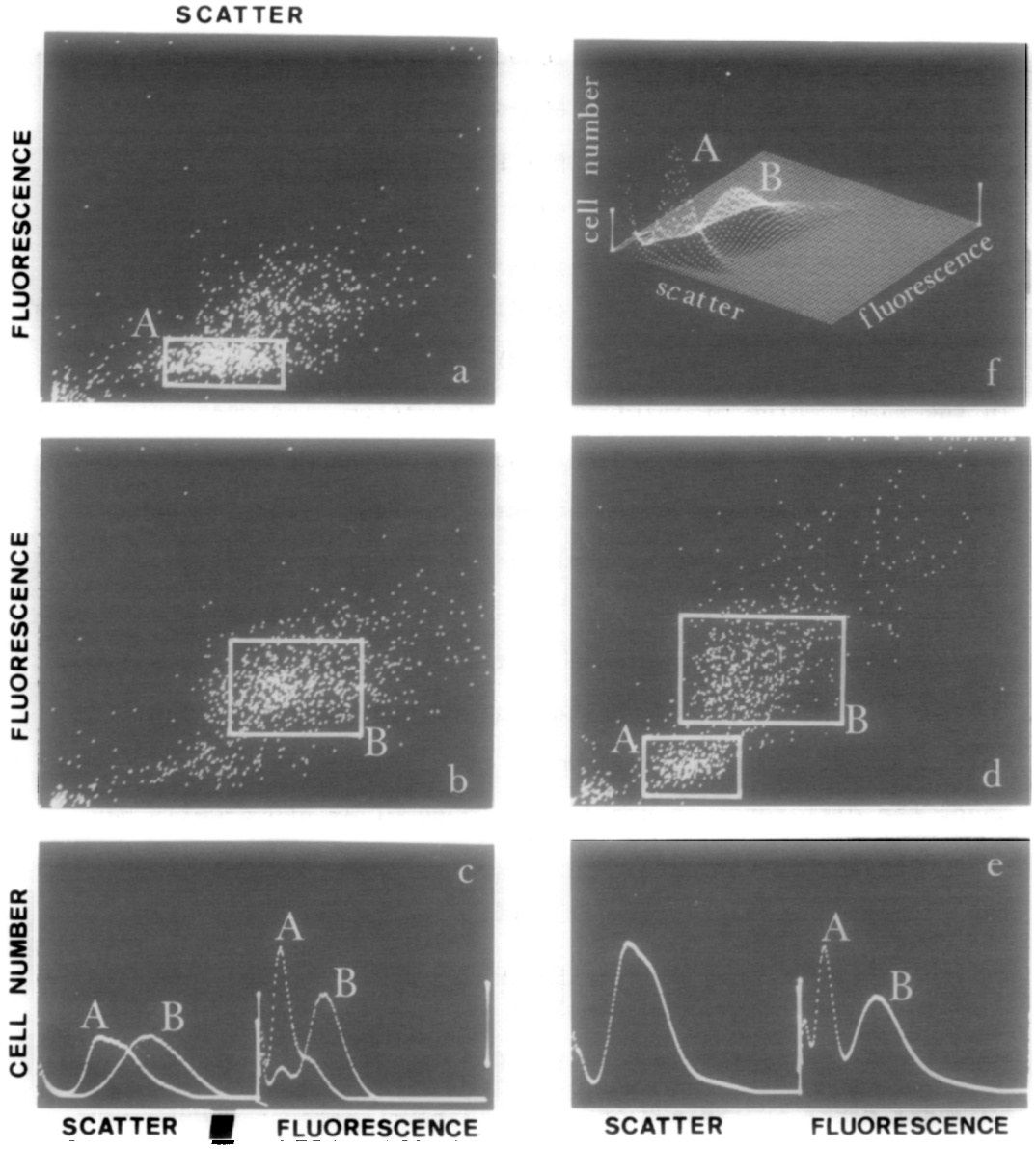

Figure 1 FACS-analysis of islet cell preparations. Comparison of the dot plots of the A-cell (a) and B-cell (b) enriched fractions led to the recognition of two different cell populations (A,B) which are represented by coinciding scatter histograms (c left panel) and differing fluorescence histograms (c right panel). Both populations were readily identified on dot plots (d), fluorescence histograms (e right panel) and isometric displays (f)of unpurified islet cells.
Sorting of A and B (squares) led to their identification as single cell fractions containing pure non-B and pure B-cells respectively; fraction A consisted of 80 to 85% A-cells, 8% D-cells and 6% PP-cells.

Table I. Relative light scatter and fluorescence intensities of various rat cell types.

Cell type		Light scatter	Fluorescence
Pituitary		690 (22)	7 (28)
Parotid		793 (23)	19 (21)
Liver		913 (22)	85 (15)
Exocrine pancreas	1	813 (12)	9 (15)
	2	834 (13)	20 (14)
Islet	A	430 (16)	4 (24)
	B	690 (13)	12 (26)
Erythrocytes		77 (23)	2 (37)
Lymphocytes		200 (10)	2 (36)

Mean light scatter intensity and mean fluorescence intensity were determined for the listed single cell populations and expressed as a percent of the intensities measured for glutaraldehyde-fixed chicken erythrocytes. Coefficients of variation are indicated between parenthesis.

pancreatic B-cells yielded comparable or higher values than other endocrine or exocrine cells (Table 1).

Characterization of cellular autofluorescence.

The fluorescence excitation (λ ex 475 nm) and emission (λ em 530 nm) spectra - recorded in TCA-hydrolysed extracts of sorted hepatocytes - coincided with those observed for the FMN-standard (Fig. 2). The emission spectrum from sorted B-cells was obtained by subtracting the spectra under reduced and oxidized conditions and was comparable to that of hepatocytes and FMN (Fig. 2).

Both sodium dithionite and potassium cyanide decreased the fluorescence intensity of B- and non-B-cells by 35 to 50%, which was also the case after a 30 min illumination.

The addition of 20 mM glucose to unpurified islet cells (37°C) decreased the autofluorescence of single B-cells by 30 to 40% with in 5 minutes; no variation was noted in the presence of 20 mM 3-0-methylglucose or fructose; under these conditions, no effects were noted upon A-cell fluorescence. A 16 hour culture period at

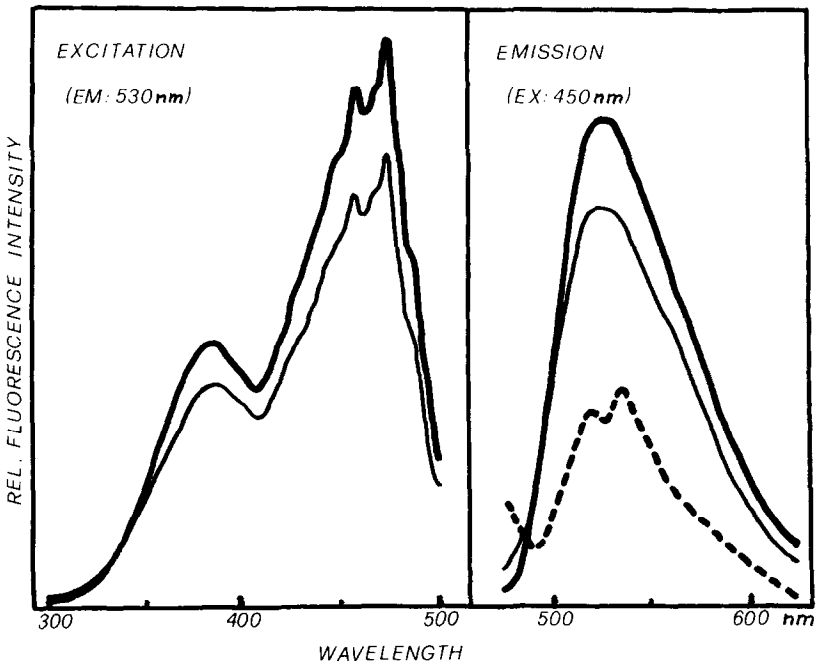

Figure 2 Fluorescence excitation and emission spectra of FAD and sorted cells hydrolyzed in TCA :
—— FAD (0.8 µg/ml - amplif. 100x)
▬▬ hepatocytes (4.10^6/ml - amplif. 50x)
--- islet B-cells ($6,5.10^5$ cells/ml - amplif. 5000x).

5.6 mM glucose and 37°C doubled the fluorescence intensity of both B- and A-cells as compared to freshly isolated cells.

DISCUSSION

The lack of methods for the purification of pancreatic A- and B-cells constitutes a major obstacle in diabetes research. The feasibility of separating both cell types appeared from their marked difference in cell size and was first demonstrated by counterflow centrifugation (2). In an attempt to prepare highly purified A-cells, we have tested whether the differences in islet cell size are associated with differences in light scatter and autofluorescence and might thus lead to a fast, visually monitored islet cell purification by fluorescence-activated cell sorting.

The finding that A-cells scatter less light than B-cells is consistent with the notion that light scatter varies with cell size (10); however, the scatter histogram of unpurified islet

cells did not distinguish clearly between both cell types and is thus by itself not a useful basis for islet cell purification, as is also evident from a recent report (11). It is known from other cell systems that minor differences in light scatter become a useful parameter in cell sorting when they are associated with differences in cellular fluorescence (12). Since viable mammalian cells produce an FAD-related autofluorescence by excitation at 488 nm (13) and since islet cells contain high flavin levels (4,5), we have measured the A- and B-cell fluorescence under these conditions. Single A-cells were 3-fold less fluorescent than single B-cells, which made both populations easily distinguishable in FACS-dot-plots of unpurified islet cells. Islet cell sorting thus succeeded in the complete isolation of single A and B-cells, which makes both cell types available for a direct (sub)cellular investigation; further studies are also facilitated by the visual selection of sorting criteria, as this feature eliminates contamination by debris and dead cells and minimizes variations in cellular composition of the sorted fractions.

The immediate display of light scatter and fluorescence intensities of single A- and B-cells can also be used for fast comparative analytical studies. Islet cell culture at basal glucose levels increased both A- and B-cell fluorescence which can be correlated with their increased secretory capability (3). A 5 min exposure to 20 mM glucose - known to stimulate insulin release - selectively decreased B-cell autofluorescence, an effect which was not observed with equal amounts of non-metabolized and non-stimulating monosaccharides. FACS-analysis of islet cells can thus contribute in differentiating the glucose effects upon the various islet cell types.

Previous studies have documented that the fluorescence measured under the present conditions originates in cellular FAD (4,5,13,

14); this notion is supported by the presently observed fluorescence characteristics and by the finding that this fluorescence decreased after light exposure and after reduction. FAD fluorescence intensity varied widely for the different cell types examined, making it an attractive parameter for their purification and their metabolic analysis. Although these applications were not in the scope of our study, the present experiments do indicate that the procedure used for islet cells also allows the purification of viable and single cells from other tissues.

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REFERENCES

1. Unger, R.H., and Orci, L. (1981) N. Engl. J. Med., 1518-1523.
2. Pipeleers, D.G., and Pipeleers-Marichal, M.A. (1981) Diabetologia 20, 654-663.
3. Pipeleers, D.G., In't Veld, P., Maes, E., and Van De Winkel M. (1982) Proc. Natl. Acad. Sci. (USA).
4. Panten, U., and Ishida, H. (1975) Diabetologia 11, 569-573.
5. Mac Donald, M.J. (1981) Endocrinology 108, 1899-1902.
6. Lacy, P.E., and Kostianovsky, M. (1967) Diabetes 16, 35-39.
7. Buitrago, A., Gylfe, E., Heriksson, C., and Pertoft, H. (1977) Bioch. Biophys. Res. Comm. 79, 823-828.
8. Herzenberg, L.A., Sweet, R.G., and Herzenberg, L.A. (1976) Scient. Am. 234, 108-117.
9. Cairns, W.L., and Metzler, D.E. (1971) Chem. Soc. 93, 2772-2777.
10. Visser, J.W.M., Van Den Engh, G.J., and Van Bekkum, D.W. (1980) Blood cells 6, 391-407.
11. Nielsen, D.A., Lernmark, A., Berelowitz, M., Bloom, G.D., and Steiner, F.D. (1982) Diabetes 31, 299-306.
12. Watt, S.M., Burgess, A.W., Metcalf, D., and Battye, F.L. (1980) J. Histoch. Cytochem. 28, 934-946.
13. Benson, R.C., Meyer, R.A., Zaruba, M.E., and McKahn, G.M. (1979) J. Histoch. Cytoch. 27, 44-48.
14. Aubin, J.E. (1979) J. Histoch. Cytoch. 27, 36-43.