Rapid Publication

Sorting Human β-Cells Consequent to Targeted Expression of Green Fluorescent Protein

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Pancreatic islets of Langerhans are composed of four major endocrine cell types with a smaller number of nonendocrine cells. To study the molecular constituents and function of just one subpopulation of islet cells, it is necessary to sort them from the other cell types. While rat β-cells can be sorted by autofluorescence-activated flow cytometry, this has not proved possible on a routine and reproducible basis for human β-cells. In the present study, we have selectively labeled human β-cells with green fluorescent protein (GFP), allowing for their sorting by flow cytometry. Human islet cells were infected with replication-defective (attenuated) recombinant adenovirus expressing GFP driven by the rat insulin I promoter (Ad-RIP-GFP) for targeted expression in β-cells, or β-galactosidase driven by the promiscuous cytomegalovirus (CMV) promoter (Ad-CMV-β-gal) as control. Whereas the majority of islet cells can be infected by adenovirus, as shown by control infection with Ad-CMV-β-gal, increased fluorescence after infection with Ad-RIP-GFP was limited to insulin-containing β-cells. Infection of islet cells with Ad-RIP-GFP resulted reproducibly in the appearance of a population of intensely fluorescent cells, when analyzed by flow cytometry. These cells were sorted using a fluorescence-activated cell sorter (FACS) and shown by immunofluorescence to consist of >95% β-cells. The targeted expression of GFP thus allows for preparation of human β-cells purified close to homogeneity. This method should be readily applicable in any laboratory with FACS capability. *Diabetes* 47:1974–1977, 1998

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Ad-CMV-β-gal, promiscuous cytomegalovirus promoter; Ad-RIP-GFP, rat insulin I promoter; BSA, bovine serum albumin; CMV, cytomegalovirus; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FSC, forward scatter; GFP, green fluorescent protein; KRBB, Krebs-Ringer bicarbonate buffer; moi, multiplicity of infection; PBS, phosphate-buffered saline; pfu, plaque-forming unit; SSC, side scatter.

Islets from most species, including humans, consist of ~75% β-cells. Whereas insulin synthesis and secretion is limited to β-cells and can thus be studied in intact islets, understanding the molecular machinery underlying these functions or the metabolic events allowing for their regulation by glucose is complicated by the presence of non–β-cells in the mixed islet cell population. To circumvent this problem, Van de Winkel et al. (1) established an elegant technique for sorting islet cells into discrete populations, including one highly enriched in β-cells, based on intrinsic cellular autofluorescence. While this technique has proven to be applicable on a routine and reproducible basis to rat islet cells in a number of laboratories, including our own (2), this has not been the case, at least in our hands, for human islet cells.

The strategy favored to circumvent this problem has capitalized on a feature unique to β-cells to sort them from non–β-cells, that is, insulin gene expression. This has been coupled to two tools of contemporary cell and molecular biology: recombinant adenovirus as a vector for expressing a foreign gene in primary (nonreplicating) cells (3) and green fluorescent protein (GFP) as a fluorescent tag for living cells (4,5). The strategy depends on infection of human islet cells with replication-deficient (attenuated) recombinant adenovirus expressing GFP under the control of the insulin promoter, thereby restricting GFP expression to β-cells that can then be sorted by flow cytometry on the basis of their GFP fluorescence. The results of this study demonstrate that this novel sorting strategy allows for preparation of highly purified human β-cells.

RESEARCH DESIGN AND METHODS

Human islet cells. Islet isolations were performed in the Division of Surgical Research at the University Hospital of Geneva. Whole pancreases including duodenum were harvested from four multi-organ heart-beating donors (23–55 years) after hypothermic perfusion via the abdominal aorta and the inferior mesenteric vein and stored in University of Wisconsin solution at 4°C in a HUG-Box (Safetherm, Geneva, Switzerland). Mean cold ischemia time before the isolation procedure was 4.5 h (2–7 h). Islets were isolated by a modified automated method as described in detail previously (6,7). Briefly, after preparation of the pancreas and distension with collagenase P (Boehringer Mannheim, Mannheim, Germany), digestion was performed at 37°C in a modified digestion chamber until the appearance of mostly free islets. Mean digestion time was 24 min (22–27 min). Islets were purified by a discontinuous Euro-Ficoll gradient (Sigma, St. Louis, MO) on a cell separator (Cobe 2991; Cobe, Lakewood, CO). Densities used were as follows: 1.108 (bottom layer), 1.096, 1.037, and 1.006 (top layer). For β-cell isolation, islets

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were harvested from both the pure second fraction (purity >80%) and from the impure third fraction (purity <50%). Only islet preparations with insufficient islet yields (<250,000), and thus unsuitable for human islet allotransplantation, were used for this study (mean islet yield 152,000 islet equivalents). Islets were cultured in CMRL-1066 medium (Sigma) supplemented with 10% fetal calf serum (FCS) (Gibco BRL, Paisley, Scotland) for up to 3 weeks at 24°C and for a further 1–2 days (immediately before viral infection) at 37°C in a humidified atmosphere of 5% CO_2/95% air. To assess islet purity, aliquots were stained (20 min; 37°C) with 1,5-diphenylthiocarbizone (dithizone from Merck, Dietikon, Switzerland) prepared as a fresh stock solution of 10 mg/ml in DMSO, diluted 1:200 in F10/FCS and filtered immediately before use. Islets are stained red by this means (8) and were shown to represent 50–80% of total cellular material. The islets used for this study were not subject to any further purification by hand-picking.

Before infection and to augment the accessibility of cells to the virus without stripping them of surface proteins implicated in adenovirus infection, islets (10–30,000) were washed two times with phosphate-buffered saline without Mg^{2+}/Ca^{2+} (PBS^{--}) and then partially dispersed by incubation at room temperature in 2 ml of enzyme-free "dissociation buffer" (catalog number C-5789 from Sigma) with frequent pipetting through a blue plastic pipette tip. The reaction was stopped with the addition of F10 medium (Gibco) containing 5% (vol/vol) FCS, 1% (wt/vol) bovine serum albumin (BSA), and 7.5 mmol/l glucose (F10/FCS/BSA). The suspension was allowed to stand for 1 min and larger aggregates sedimenting during this time were washed once in PBS^{--} and then subjected to a second round of dissociation as above. The dispersed cells (consisting of a mixture of single cells and small arrays of up to 10 cells each) were pooled, washed once in F10/FCS/BSA, and suspended in the same medium for infection with adenovirus.

Recombinant adenovirus. Recombinant replication-defective (attenuated) adenovirus-expressing GFP (S65T variant; "Green Lantern-1" from Gibco) under control of the rat insulin I promoter (Ad-RIP-GFP referred to as AdIns-GFP in [9]) was prepared as described in detail previously (9). In brief, the virus was obtained by homologous recombination in HEK293 cells cotransfected with pACRIP-GFP (referred to as pACIns.GFP in [9]) and pJM17. Virus was cloned, amplified, purified (by CsCl gradient) and stored as described previously (10). Ad-CMV-β-gal was the generous gift of Dr. Christopher Newgard (Dallas, TX) and used as described in an earlier study (10).

Virus was titered by estimating plaque formation after infection of 293 cells (10). Note, as discussed previously (10), that although routinely used for this purpose, this is not a precise method for estimating plaque-forming units (pfu). Furthermore, since the islet cells used for infection were not fully dispersed and consisted of a mixture of cell types, possibly each with a different sensitivity toward infection by adenovirus, it was impossible to count them with any great accuracy and derive a particularly meaningful estimate of multiplicity of infection (moi = pfu as a function of cell number). Values for moi are thus provided on an indicative basis. Given that high titres of adenovirus can adversely affect β-cell function (10), it is recommended that each new batch of recombinant virus be tested on human islet cells to establish the lowest titre allowing for high efficiency of infection of the β-cells.

To facilitate the use of Ad-RIP-GFP for sorting of human β-cells, we have made this recombinant adenovirus available to colleagues on written request to Dr. Larry Gene Moss (for distribution in North America) or to Dr. Philippe Halban (for distribution outside of North America).

Infection of human cells with recombinant adenovirus. Dispersed cells were suspended in F10/FCS/BSA at a concentration of 10^6 cells/ml, and 2 ml aliquots were dispensed into 20 cm^2 nonadherent Petri dishes (manufactured for bacterial culture). Ad-RIP-GFP (~2×10^8 pfu) was added to each dish to obtain a moi of ~100. The cells were incubated with virus at 37°C for 2 h, washed two times in F10/FCS/BSA, and left in culture in this medium for 48 h (2×10^6 cells/10 ml in 50 cm^2 nonadherent dishes) to allow for expression of GFP. Control cells were handled in parallel either without virus or with Ad-CMV-β-gal in place of Ad-RIP-GFP.

FACS. Two days post-infection with adenovirus, cells were harvested and treated with a solution of 0.025% trypsin (1:250 from Gibco) in PBS^{--} with 0.3 mmol/l EDTA (2–6×10^6 cells in 2 ml) for 6.5 min at 37°C (with occasional pipetting through a blue plastic tip) to obtain a dispersed cell suspension. The digestion was stopped by addition of filtered (to avoid any particulate matter for FACS) Krebs-Ringer bicarbonate buffer (KRBB) containing 10 mmol/l Hepes, 8.3 mmol/l glucose, and 0.5% BSA (KRBB-BSA). After washing two times in KRBB-BSA, cells were suspended in 3 ml KRBB-BSA, transferred to a plastic 5-ml tube, and passed 30 times in and out of a blue plastic pipette tip. They were then left to stand for 2–3 min to allow any large aggregates to sediment. The supernatant, containing mainly single cells with only occasional groups of two to three cells, was diluted to 0.8–1×10^6 cells/ml for sorting.

Cells were sorted using a FACStar-Plus (Becton Dickinson) with an argon laser beam tuned to 488 nm at 50 mW output with a 530 ± 30 nm emission filter. In biparametric plots, forward scatter (FSC) related to cell size. Presorting gates (side scatter [SSC] versus FSC) were set to exclude noncellular debris and groups of more than one cell. Noninfected cells were used as the control and to set the sorting parameters such that the GFP^+ quadrant in the biparametric plot (log fluorescence versus FSC) included <0.1% of control cells. Cells were sorted at a rate of 1,000 events per second using "normal recovery" mode.

Microscopy. Cells before sorting and the two sorted populations (GFP^- and GFP^+) were deposited (in F10/FCS/BSA) on glass microscope slides precoated with poly-L-lysine (for precoating, slides were incubated for 2 h in 1 mg/ml poly-L-lysine, molecular wt 150–300,000 from Sigma, washed in H_2O and air-dried). Cells were allowed to attach to the glass surface by incubating at 37°C for 2 h in a humidified atmosphere of 5% CO_2 and were then washed with PBS before fixation in 4% paraformaldehyde in PBS for 20 min followed by washing in PBS. The fixed cells were permeabilized with 0.2% Triton in PBS containing 0.5% BSA, incubated for 20 min with PBS/0.5% BSA, washed three times with PBS, and then kept at 2–4°C in PBS.

For immunofluorescence, cells were incubated for 2 h at room temperature with primary antibody (guinea pig anti-insulin, the generous gift of Dr. Peter Wright, Indianapolis, IN; dilution 1:400) followed by washing (three times in PBS) and 15 min incubation in PBS before incubation for 1 h at room temperature with the second antibody (rhodamine [TRITC] labeled anti-guinea pig IgG from Jackson ImmunoResearch, Westgrove, PA; dilution 1:400). After washing (three times) and 15-min incubation in PBS, the cells were preserved sealed under a cover slip in Vectashield mounting medium (Vector, Burlingame, CA) at 2–4°C in the dark.

RESULTS

Expression of GFP in human islet cells infected with Ad-RIP-GFP is limited to β-cells. Human islet cells were infected with Ad-RIP-GFP, and 48 h later the number of insulin-containing β-cells expressing GFP was monitored by combination of direct fluorescence (to visualize GFP^+) and indirect immunofluorescence to identify β-cells (Table 1 "before sorting"). All GFP^+ cells were β-cells, with no detectable $insulin^-$/GFP^+ cells in this or any other independent experiment. However, not all β-cells were GFP^+, presumably reflecting the fact that not all of them had been infected by the adenovirus, or that the level of GFP expression in some cells was too low to be detectable. The intensity of GFP fluorescence was indeed variable from one cell to the next. Approximately 75% of the GFP^+ cells were classed as "intensely" fluorescent and the remaining 25% as "modestly" fluorescent. Both groups were $insulin^+$.

Selective expression of GFP in β-cells was most logically due to cell-specific activity of the insulin promoter driving GFP in Ad-RIP-GFP. To exclude that it may have resulted from selective infection of only β-cells but not non–β-cells by adenovirus per se, islet cells were infected with Ad-CMV-β-gal, a recombinant adenovirus in which β-galactosidase expression is driven by the CMV promoter, allowing for such expression regardless of cell type. After infection with this virus, β-gal expression was seen in both β-cells and non–β-cells (not shown), confirming that both cell populations are readily infected by adenovirus.

Highly purified human β-cells are obtained by sorting GFP^+ cells. When analyzed by flow cytometry, a highly fluorescent subpopulation of cells appeared after infection with Ad-RIP-GFP, which was not apparent in control cells (Fig. 1). When these cells were sorted on the basis of their GFP fluorescence and then analyzed by immunofluorescence, it was evident that they consisted almost exclusively of β-cells (GFP^+ cells after sorting, Table 1).

Note that sorting windows (Fig. 1) were set to ensure maximum purity at the expense of cell yield. Only cells with intense fluorescence were sorted as GFP^+ in window R1 (Fig. 1). Cells expressing only modest amounts of GFP (but known to be β-cells based on their immunofluorescence, see above) thus fell outside of the GFP^+ window (R1 in Fig. 1) and were not sorted. These cells would have been heav-

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TABLE 1
Analysis of human islet cells before sorting and of the GFP$^+$ and GFP$^-$ populations after sorting by FACS

Cell population	ins$^+$	ins$^+$/GFP$^+$	ins$^+$/GFP$^-$	ins$^-$/GFP$^+$	ins$^-$/GFP$^-$
Before sorting	29.5	25.6	3.9	0	70.6
After sorting					
GFP$^+$	97.3	95.4	1.9	0	2.7
GFP$^-$	14.9	8.1	6.8	0	85.1

Data are percentage of total cells observed. GFP$^+$ and GFP$^-$ populations are those represented in Fig. 1 as R1 and R2, respectively. Insulin-containing cells (ins$^+$) were identified by immunofluorescence using anti-insulin antiserum. Data are from one representative experiment out of four independent experiments.

ily contaminated with cells not expressing GFP (compare the zone lying between windows R1 and R2 in Fig. 1*A* and *B*). It was also found important to analyze and sort only cells pre-gated on the basis of side and forward scatter (see METHODS and Fig. 1). This limited contamination of the GFP$^+$ population with other intrinsically fluorescent cells, aggregates or debris, to an acceptable minimum (<0.1% of uninfected cells thus fell in the GFP$^+$ sorting window, Fig. 1*A*). It is estimated that ~50% of β-cells are lost in the application of such stringent sorting criteria. When this value is combined with the percentage of β-cells in a typical starting preparation of unsorted cells derived by dispersion of human islets without hand-picking, as well as the inevitable losses encountered when sorting by flow cytometry (maximum theoretical yield ~80%), it can be estimated that ~10% of the total starting cell number may be recovered in the purified β-cell population. Given the numbers of human islets that can be obtained from a single pancreas, this is not an obstacle to obtaining usefully large numbers of β-cells.

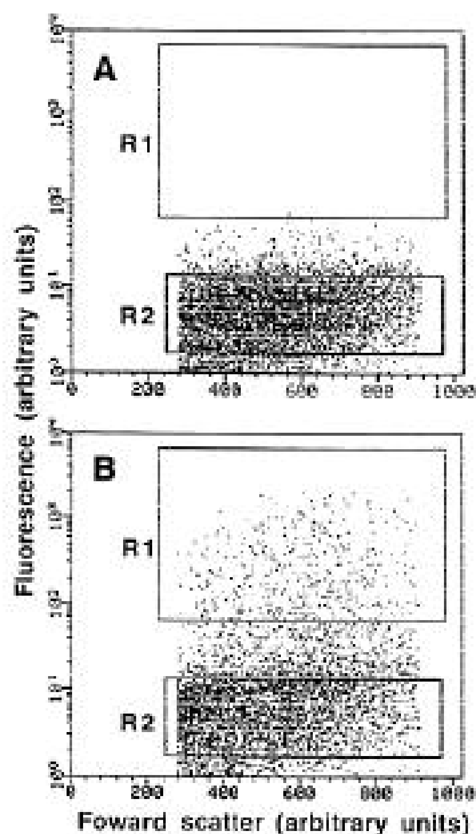

FIG. 1. Biparametric cytometric analysis of human islet cells. Fluorescence (logarithmic scale), reflecting cellular GFP content, is plotted against FSC (linear scale), reflecting cell size. Presorting gates were set (SSC versus FSC; not shown) to include only single viable cells (representing 56% of total nongated events) in the biparametric analysis used for sorting. *A*: Uninfected cells; *B*: cells 48 h after infection with Ad-RIP-GFP. Sorting windows were set as indicated (▭). R1 was taken as the GFP$^+$ population and R2 as GFP$^-$. For control and infected cells, 0.04 and 13%, respectively, of gated events were in R1. Data are from one representative experiment out of four independent experiments.

The data presented in Fig.1 and Table 1 are from one out of a total of four independent experiments in a recent series using islets isolated in Geneva. These experiments, however, followed from a larger series of preliminary studies on a total of 31 islet preparations obtained from alternative sources (Miami and Brussels, see ACKNOWLEDGMENTS). These preliminary experiments allowed for the final protocol to be established. For the final series of experiments, there was considerable variability from one islet isolation to the next in terms of the purity of the preparation (see METHODS) and the percent of β-cells expressing GFP after viral infection, preventing interpretable presentation of mean data for all experiments. Thus, the unsorted cell population was composed of 27–54% β-cells (a reflection of the purity of the islets), while 56–93% of β-cells expressed GFP after infection with Ad-RIP-GFP. However, variability in these parameters had no impact on the purity of the sorted β-cell population, which was reproducibly in excess of 95% when using the sorting windows and gates described above and in Fig. 1. Furthermore, and most significantly, no GFP$^+$ non–β-cells were encountered in any experiment, highlighting the specificity of GFP expression.

The β-cells purified to date have not been analyzed further to assess their function. However, the purified cells did attach and spread on an extracellular matrix derived from 804G cells (11) (data not shown), which is considered a sensitive index of β-cell viability and integrity (12), particularly when purified β-cells are plated in the absence of contaminating fibroblasts, which can themselves promote β-cell attachment (13).

DISCUSSION

The availability of highly purified rat β-cells has allowed for detailed biochemical analysis of insulin-producing cells and their comparison with non–β-cells. Given that there are data suggesting major differences between human and rat β-cells (as exemplified by respective levels of the transporter GLUT2 [14]), it will be important to gain access to a

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similarly pure preparation of human β-cells. Using purified β-cells would represent a significant advantage over mixed, nonsorted, islet cells, for a number of other applications, including the as-yet-elusive preparation of a human β-cell cDNA library. This will now be possible based on the present method, which allows for preparation of β-cells purified essentially to homogeneity (>95% purity).

It remains unclear why sorting by autofluorescence, which has proven so successful for rat islet cells, is not so readily applicable to human cells: in our own hands this approach has never proved successful for purifying human β-cells. One published report provides some evidence for successful sorting but no detailed quantification of purity or reproducibility (15), while others have found a variable degree of enrichment of human β-cells (80–95% purity) by autofluorescence sorting, so long as islets are hand-picked before dissociation and flow cytometry (Dr. Vincenzo Cirulli, Whittier Institute, San Diego, CA, personal communication). Sorting by autofluorescence depends on differential intermediary metabolism (flavin adenine dinucleotide [FAD] levels) in β-cells versus non–β-cells (1). In the present study, sorting of β-cells is based on the best-documented and most fundamental differentiated function of this cell type, the ability to express the insulin gene. The cells were, however, infected with virus. Even recombinant, replication-deficient (attenuated) adenovirus, used to infect the islet cells, can modify cell function when high viral titers are used for infection (9,10,16). The relatively low titer used in the present experiments has been shown previously for this particular RIP-GFP virus not to affect glucose sensitivity of insulin gene expression in infected β-cells and, based on the use of other recombinant viruses, is not expected to result in modification of other aspects of β-cell function (10,16–18). Regardless, the presence of some viral proteins and GFP in the sorted cells is an unavoidable facet of this methodology, which may preclude their use for certain applications.

Although not tested here and given the known functional heterogeneity of rat β-cells (19), it will be interesting in future experiments to determine whether advantage can be taken of the known sensitivity of the insulin promoter to glucose (as confirmed using the very same adenovirus as in this study [9]) to purify a subpopulation of β-cells uniquely sensitive to glucose. Such cells would be expected to exhibit enhanced GFP fluorescence (resulting from increased GFP production driven by glucose stimulation of the promoter) after incubation at high glucose.

In conclusion, infection of islet cells with adenovirus expressing GFP under the control of the insulin promoter allows for targeted expression of GFP in β-cells and for their subsequent sorting by FACS. A highly enriched population of β-cells can be obtained even when using human islets purified only by density-gradient centrifugation (i.e., without hand-picking), a prerequisite for use of large numbers of islets as starting material for sorting. This method should be readily applicable in any laboratory with FACS capability, and the recombinant adenovirus will be made available to the research community for this purpose (see METHODS). The strategy employed here is not restricted to pancreatic β-cells. On the contrary, it should be applicable to FACS of any cell type that displays selective expression of a gene for which the promoter has been characterized and which can be used to control GFP expression in place of the insulin promoter used here.

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