Analysis of insulin receptors on heterogeneous eukaryotic cell populations with fluorochrome-conjugated insulin and fluorescence-activated cell sorter. Advantages and limitations to the 125I-labelled insulin methodology

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Summary. The diversity in insulin receptor expression within eukaryotic cell populations can be studied with fluorochrome conjugated reagents with high affinity to the insulin receptor in combination with flow cytometry. We studied the optimal conditions for application of fluorescein isothiocyanate (FITC) conjugated insulin in combination with the fluorescence activated cell sorter (FACS) to analyse insulin receptor expression, and also studied the feasibility of this method for identifying and isolating viable subsets with differences in insulin receptor expression within a cell population. Semisynthetic human insulin was conjugated to FITC, which resulted in at least four types of FITC-insulin molecules with different affinities to the insulin receptor. Each type of FITC-insulin was isolated by semipreparative reverse phase high pressure liquid chromatography. The preparation with a fluorescein/protein ratio of ~1.0 was found to have the highest affinity to the receptor, the highest biological activity (~50% of native insulin), and similar antigenicity as native insulin. The optimal staining conditions with respect to pH, time of incubation, and cell number were determined, and were different in some aspects from labelling with 125I-insulin. The binding of FITC-insulin to cells was saturable and could be displaced with unlabelled insulin. The fluorescence signal could be converted to absolute numbers of fluorescein molecules by a calibration curve, and the absolute number of specifically bound FITC-insulin molecules calculated from a F/P ratio ~1.0. The FITC-insulin/FACS method permits estimation of the total number of insulin receptors (high plus low affinity), and the data obtained correlate well with the results from Scatchard plot of 125I-insulin binding data. However, the latter method also permits selective detection of the number of high affinity insulin receptors, which cannot be done with FITC-insulin/FACS that has a lower level for detection of fluorescence on ~2000–3000 fluorescein molecules per cell. The FITC-insulin/FACS methodology permitted identification and isolation of viable cellular subsets within a cell population as based on the number of insulin receptors and was also used to study variations in insulin receptor density in human leucocytes. The method should make it possible to perform a number of hitherto unfeasible analyses of the biology of insulin receptor expression on eukaryotic cells.

Key words: Insulin receptors, fluorescein-conjugated insulin, fluorescence activated cell sorter analysis, heterogeneity in insulin receptor density.

Phenotypic diversification has been described within clonal derived populations of both normal and malignant cells [1]. Identification and isolation of subsets of cells with respect to a cell constituent should in principle be feasible by fluorescence activated cell sorter (FACS) analysis/sorting of cells stained with a fluorochrome conjugated reagent with high binding affinity for the constituent. Antibodies with specificity for cell membrane antigens have in particular been used as such reagents, and this has been reinforced by the development of monoclonal antibody technology [2].

Expression of hormone receptors has typically been estimated by Scatchard plot [3] of the data for binding of radiolabelled hormone to the cells, although Scatch-

ard analysis only has limited value in a number of situations [4]. Moreover, a curvilinear Scatchard plot is consistent with either multiple classes of insulin receptors or site-site interactions of the type known as negative cooperativity [5]. However, it might also reflect the presence of subsets of cells with different receptor density within the cell population. Heterogenicity in insulin receptor expression cannot be explored with radiolabelled ligand, which measures the average receptor expression of the cell population. It is accordingly of importance to develop methods that permit identification and further investigation of subsets of cells with different hormone receptor expression within a cell population.

Fluorochrome (e.g. fluorescein isothiocyanate, FITC) conjugated lectins have been used in combination with flow cytometry to evaluate their binding to cells [6, 7]. However, the conditions for application of FITC-lectins, e.g. FITC-concanavalin A, cannot readily be used for specific hormones like insulin, partly because the biochemical structures of lectins are very different from peptide hormones, and partly because a lectin like concanavalin A binds to a number of different glycoproteins with approximately the same affinity, whereas a peptide hormone binds with high affinity only to its specific receptor.

A few reports have described the use of FITC [6, 8] or rhodamin [9, 10] conjugated insulin. Some of these analyses have also applied flow cytometry. However, none of these reports give detailed data for the conditions for the labelling of cells with FITC-insulin (e.g. pH, incubation time), the structural alteration of insulin by FITC-conjugation (e.g. altered antigenicity). In addition, no direct comparison between FITC-insulin data and ^{125}I-insulin data has been done. A few of the reports [8, 9, 10] have indicated that fluorochrome conjugation resulted in reduction of the biological activity of insulin to ~1.15% of native insulin. High purification of FITC-insulin with high biological activity and binding affinity seemed therefore desirable prior to its use in combination with flow cytometry (FITC-insulin/ FACS).

We have, therefore, purified FITC conjugated insulin by reverse phase high pressure liquid chromatography and obtained a FITC-insulin preparation with a fluorescein/protein (F/P) ratio ~1.0 and with ~50% biological activity and binding affinity as compared to native insulin. The conditions for staining of cells with FITC-insulin (pH, incubation time, cell number) and the antigenicity of FITC-insulin as compared to native insulin is described. The total number of insulin receptors on various eukaryotic cells was measured by FITC-insulin/FACS and by ^{125}I-insulin labelling, respectively, and the feasibility of the FITC-insulin/FACS method to identify subsets of cells with different insulin receptor expression was demonstrated in a population of mononuclear blood leucocytes (lymphocytes and monocytes). The FITC-insulin method could be applied both to fixed and viable cells.

Materials and methods

Sources of cell material

Peripheral blood leucocytes were obtained from 20–40-years-old volunteers in the laboratory. The mononuclear cells were isolated on a Ficoll-hypaque gradient and washed free of insulin as previously described [11] before incubation with FITC-insulin. No attempts were made to isolate the monocyte fraction prior to staining, as monocytes could be distinguished from lymphocytes by the FACS scatter signals [12]. Viability as assessed by trypan blue dye exclusion was >95%, and pH remained stable during the incubation period.

Human cell lines were: U-937 (histiocytic lymphoma; [13]), HL-60 (promyelocytic leukemia; [14]), RH-L4 (B-lymphoma; [15]), Daudi (Burkitt lymphoma; [16]) and IM-9 (lymphoblastoid cells; [17]). All lines were maintained in RPMI-1640 medium with 10–15% fetal calf serum (FCS), supplemented with 0.3% fresh glutamin, at 37 °C with 95% atmospheric air and 5% CO_2. Only cells in the exponential growth phase were used.

In some experiments, spleen cells from 4–6 week-old female Balb/c mice were used. The mice were obtained from our own specific pathogen-free colony and maintained under standard conditions with water and food ad libitum.

Hormone preparations

Semisynthetic human insulin, ^{125}I-conjugated insulin (conjugated at Tyr-A14: specific activity 200–300 μCi/μg), purified A- and B-chains of insulin, and glucagon were all obtained from Novo Industry A/S, Bagsværd, Denmark. FITC (Sigma, lot No.102F5045) conjugation was done at room temperature by the following procedure: 100 mg to 1 g insulin was dissolved in phosphate buffered saline (PBS) to a concentration of 10 mg/ml and the solution adjusted to pH 9 with a 0.5M bicarbonate/carbonate buffer. FITC was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 4 mg/ml and added dropwise under constant stirring to the insulin solution (5 μl FITC per mg insulin). The mixture was incubated in the dark for 2 h and free FITC separated from insulin and FITC-insulin on a G-25 sephadex column (Pharmacia, Uppsala, Sweden) equilibrated with PBS (pH 7). Fifty to one hundred mg insulin and FITC-insulin were separated into five fractions by semipreparative reverse phase HPLC on a 10 mm × 250 mm column of 7 μ C-18 derivatised silica (Merck No.50994). The column was eluted at 6 ml/min with 0.2 M $(NH_4)_2SO_4$, pH 3, using a gradient from 20% to 45% acetonitrile during 20 min. Unchanged insulin eluted at 15.8 min followed by four different fluorescein labelled insulins eluting at 17.8 min, 18.3 min, 19.2 min, and 21.0 min, respectively. The fractions were collected, the acetonitrile removed on a rotary evaporator, and the remaining solution adjusted to pH 7. The fraction eluting at 19.2 min had a F/P ratio of 0.98 as estimated from spectrophotometric measurements at 280 nanometers (peptide) and at 495 nm (fluorescein). And FITC is under these conditions almost exclusively conjugated to phenylalanin in the B-chain (B1) [18]. This fraction had the highest affinity to insulin receptors, was displacable with native insulin, and had high biological activity (see below). It was therefore used in all experiments.

Labelling of cells with FITC-insulin or ^{125}I-insulin

One million cells were suspended in 300 μl PBS containing FITC-insulin (0.1 μg/ml unless otherwise stated) for up to 120 min at 17 °C. In some experiments cold insulin was added in various concentrations. The incubation was done on a rocking platform to avoid sedimentation and was terminated by washing the cells twice in ice cold PBS with subsequent fixation in a 0.5% formaldehyde solution. The staining pattern remained stable for 7–14 days after fixation, when the cells were stored at 0–4 °C (Fig. 1). In some cases, the cells were FACS analysed immediately after staining and thus without fixation. Formaldehyde fixation did not change fluorescence intensity as compared to viable cells.

The assay with ^{125}I-insulin was performed as described elsewhere [19]. One to six million cells per ml were incubated in RPMI-1640 medium, pH 7.6, with 20 pM ^{125}I-insulin and in the absence or presence of unlabelled insulin in concentrations from 10 pM to 3 μM. Nonspecific insulin binding was defined as the amount of ^{125}I-insulin bound to cells in the presence of a large excess (3 μM) of unlabelled insulin and did not exceed 2%. All samples were run in triplicates.

Assay for biological activity of insulin

The biological activity of semisynthetic human insulin and FITC-insulin was measured by incorporation of ^{3}H-deoxy-glucose into lipids of isolated fat cells [20].
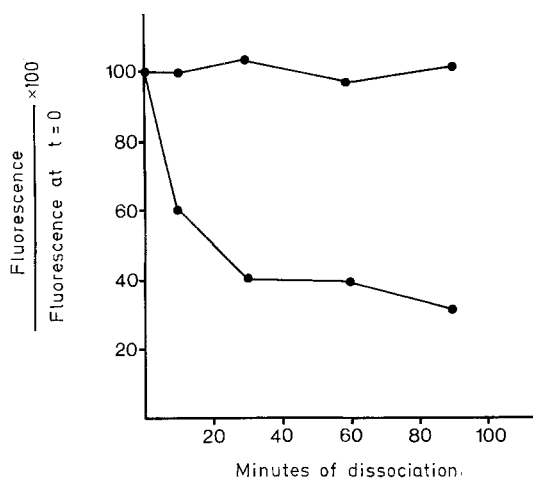
Fig. 1. Effect of fixation in 0.5% formaldehyde on the dissociation of FITC-insulin. FITC-insulin 0.1 µg/ml was incubated with U937 cells (3.33 × 10⁶/ml) for 90 min at 17 °C and pH 7. The cells were washed twice in PBS. Some cells were fixed in 0.5% formaldehyde (O) and other cells were resuspended in PBS (●). Four tubes of each set were analysed by FACS for each time point. The mean fluorescence on cells, expressed as a percentage of the fluorescence at time (t) = O is plotted as a function of time. Each point is the mean of quadruplicates which differed less than 12%
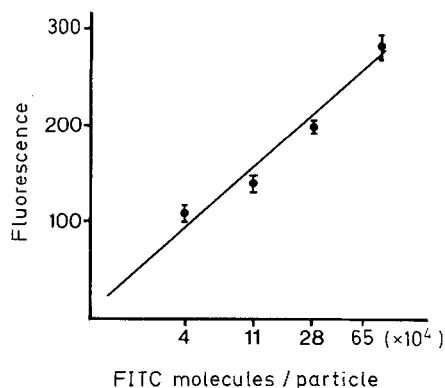

FITC molecules / particle

Fig. 2. Calibration curve for the relationship between the fluorescence channel number and number of fluorescein molecules. Latex particles with a known mean number of FITC-molecules per particle were FACS analysed and the mean channel number plotted as a function of fluorochromes per particle. The curve was constructed as the best fit of a straight line to the experimental data by the least square method. Each value represents mean of triplicate measurements. Bars represent SD

Anti-insulin antibodies

Polyclonal antibodies against insulin of human, porcine, and bovine insulin were obtained from Novo Industry A/S, Bagsværd, Denmark. Human anti-insulin receptor antibodies [21] were provided from Dr. J. Nerup, Steno Memorial Hospital, Gentofte, Denmark.

Murine monoclonal antibodies were generated against insulin. Briefly, 4–6-week-old Balb/c mice were immunized with bovine insulin (15 µg s. c. per injection). Four days after the last of five injections, mononuclear spleen cells were fused with the X63. Ag8.6.5.3 murine myeloma cells according to procedures described previously [15]. Supernatants were screened for reactivity to bovine, porcine, and semi-synthetic human insulin by ELISA, positive cultures were cloned twice by limiting dilution, and monoclonality of relevant cultures as-

sured by 2D-analysis of ³⁵S-methionine labelled Ig-products. Seven hybridoma clones with reactivity against insulin were established as ascites tumours in mice, and Ig purified from the ascites fluid.

The reactivity of the polyclonal antibody to insulin and FITC-insulin was analysed in an Ouchterloney test [22]. The reactivity pattern of the monoclonal antibodies was tested by ELISA in a 96-well plate. Plates coated with 1 µg per well of insulin or FITC-insulin were incubated for 3 h at room temperature with 10 µg antibody. Free sites were coated with 1% FCS/PBS. The plate was washed five times in PBS, and rabbit anti-mouse Ig conjugated with alkaline phosphatase used as second step reagent.

Light and electron microscopy

Cytocentrifuge preparations were made of cells and stained with Giemsa-Wright stain to test for morphological alterations as a function of exposure to insulin. Cells for electron microscopy were centrifuged to a pellet, fixed in 2% glutaraldehyde, and processed. Incubation of cells with insulin (10 µg/ml) and FITC-insulin (0.1–0.5 µg/ml) did not result in morphological alterations of the cells.

FACS analysis

All FACS analyses were performed with a FACS IV (Becton-Dickinson, Mt. View, Ca.). The instrument operates with an argon-laser (Model 164-05, Spectraphysics), which was used with a wavelength at 488 nm. A neutral density filter (1%) was used for the scatter signal and for the fluorescence signal a 530-nm filter. Five or ten thousand cells were analysed per sample at a rate of 500–3000 cells per second and with a nozzletip with 80 µm in diameter. The trigger signal was determined by the scatter signal. A program was developed that permits transfer of all scatter and fluorescence signals for each cell to a HP 9836 computer (Hewlett-Packard, Palo Alto, Ca), which permits a detailed data analysis of subpopulations, whenever needed. A calibration curve for the relationship between the FACS signal as measured by arbitrary channel number, and the absolute number of FITC molecules was established every time a set of cell samples was analysed. The following FITC conjugated latex particles (provided by Becton Dickinson, Mt. View, Ca) were used: (1) bead diameter 4.3 µm, scatter cv 2%, fluorescence cv 10%, 4.2 × 10⁴ FITC molecules/bead; (2) bead diameter 5.3 µm, scatter cv 2%, fluorescence cv 10%. 10.8 × 10⁴ FITC molecules/bead; (3) bead diameter 6.3 µm, scatter cv 2%, fluorescence cv 10%, 2.8 × 10⁵ FITC molecules/bead; (4) bead diameter 7.8 µm, scatter cv 2%, fluorescence cv 10%; 6.5 × 10⁵ FITC molecules/bead. Non-fluorescent beads with a diameter of 6.3 µm, scatter cv 2%, were also used. The calibration curve should theoretically be a straight line, which was confirmed experimentally (Fig. 2). The establishment of a calibration curve prior to each analysis of a set of samples also permitted the evaluation of short- and long-term FACS drifting, which was found to be insignificant.

Results

FITC-insulin binding in relation to cell number, pH, and time

The nonspecific binding of FITC-insulin was estimated by the fluorescence signal of cells stained with FITC-insulin in the presence of an excess (10 µg/ml) of unlabelled insulin (Fig. 3). The mean number of FITC-molecules per cell was derived from the calibration curve (Fig. 2) that for a given fluorescence intensity as indicated by channel number allows conversion of the channel number into an absolute number of fluorescence molecules. The number will also be the number of bound
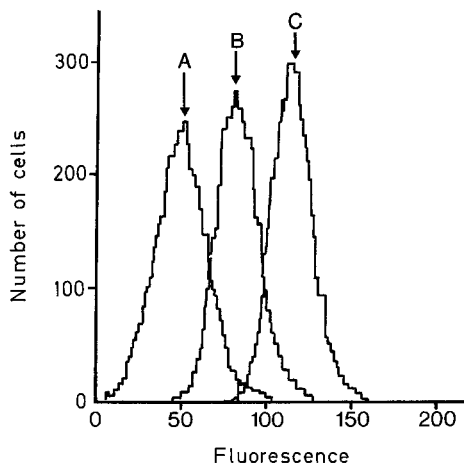
Fig. 3. Fluorescence signals from HL60 cells stained with FITC-insulin (0.1 μg/ml) for 90 min at 17 °C. The curves are: (A) unstained cells, (B) cells stained with FITC-insulin in the presence of native insulin (0.1 mg/ml), (C) cells stained with FITC-insulin in the absence of native insulin. The fluorescence intensity is given in arbitrary channel numbers; each histogram represents the analysis of $\sim 10^4$ cells

Fig. 4. pH dependence of binding of FITC-insulin to U937 cells. Cells were stained with FITC-insulin (0.1 μg/ml) at various pH values, fixed in formaldehyde, and FACS analysed. The mean channel numbers were converted to absolute number of fluorescein molecules. The fluorescence is given in per cent of fluorescence at pH 6.5. The dotted line represents unspecific fluorescence. Each point is the result of 2 separate experiments ($n = 8$). Bars indicate SD

FITC-insulin molecules, because F/P ratio is ~ 1.0 and the specific binding is determined as number of FITC molecules bound in the absence of native insulin minus number of FITC molecules bound in the presence of high saturating concentration of native insulin.

The binding of a tracer amount of ^{125}I-insulin has been shown to be a linear function of the cell concentration [19]. By incubating various concentrations of cells with 1.0 μg/ml FITC insulin in 300 μl PBS, pH 7, no effect of the concentration of cells was observed on the staining intensity of the individual cells in a cell number range of 2×10^5 to 30×10^5 cells/300 μl. The dosis of 1 μg/ml of FITC-insulin was thus at the saturation level, and the number of FITC-insulin molecules bound to a given cell population was therefore directly proportional to the total number of cells in the population. Thus, a peak of fluorescence in channel No. 100 of 10^6 cells and also in channel No. 100 of 2×10^6 cells indicate that the number of FITC molecules bound to 2×10^6 cells is twice the number bound to 10^6 cells. This is in full accordance with ^{125}I-insulin data obtained by others [19]. The pH optimum for binding of FITC-insulin was found to be between 6.5–7.0 (Fig. 4), which is lower than pH optimum for binding of ^{125}I-insulin [5]. Figure 5 shows the specific binding of FITC-insulin as a function of time at 17 °C. Steady state was obtained after about 60 min and maintained for 30 min.

Displacement of FITC-insulin by unlabelled insulin

The binding of FITC insulin was displacable with unlabelled insulin (Fig. 6). Slightly more than 60% of the total fluorescence could be displaced by incubation of cells with native hormone at 10 μg/ml. Glucagon and purified A- and B-chains of insulin had no effect on

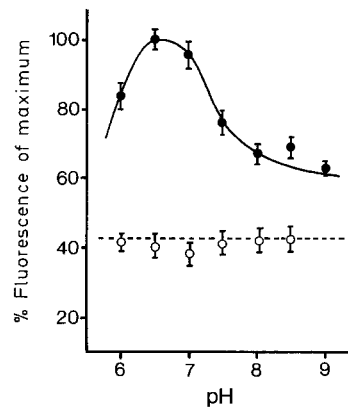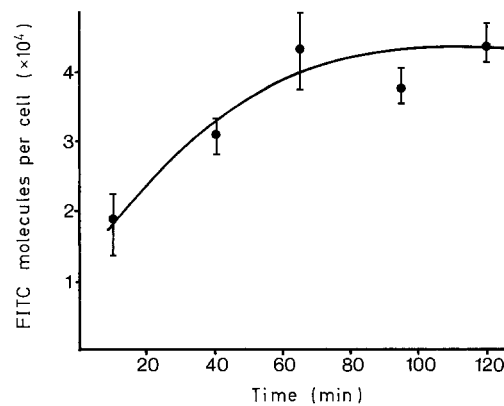**Fig. 5.** Specific binding of FITC-insulin to U937 cells as a function of time. U937 cells ($10^6/500$ μl) were incubated with FITC-insulin (0.1 μg/ml) in the presence or absence of unlabelled human insulin (10 μg/ml). Cells were analysed by FACS at different time intervals and the specific binding calculated. Each point represents the mean of two experiments. Bars represent SD

FITC-insulin binding. It cannot be completely excluded that a proportion of the 40% unspecific FITC-insulin was due to internalisation of FITC-insulin, although all experiments were carried out at 16–17 °C.

Antigenic properties of FITC-insulin

FITC-insulin and native insulin were compared by Ouchterloney analysis using a panel of 9 antisera against insulin of human, porcine, and bovine origin. FITC-insulin and native insulin precipitated identically with 8 of 9 antisera. One serum precipitated only the unlabelled hormone. ELISA-tests with monoclonal antibodies directed against insulin did not show any antigenic differences between FITC-insulin and semisyn-
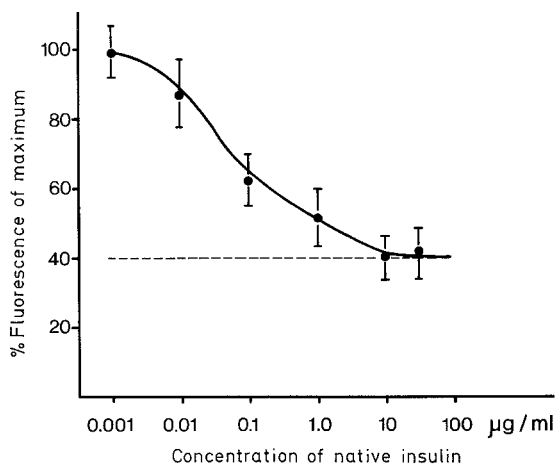
Fig. 6. Inhibition of FITC-insulin binding by unlabelled insulin. 10^6 U937 cells were incubated for 90 min at 17 °C with 0.1 μg/ml FITC-insulin in the presence of unlabelled insulin. The cells were fixed in formaldehyde prior to FACS analysis. The mean fluorescence channel numbers were converted to absolute numbers of fluorescein molecules. The fluorescence is given in per cent of maximal binding after subtraction of the fluorescence of unlabelled (autofluorescence) U937 cells. The dotted line indicates unspecific binding. Each point is the mean of 3 separate experiments ($n = 12$). Bars indicate SD

thetic human insulin. The elution patterns of FITC-insulin and native insulin through columns of sepharose, conjugated with monoclonal antibodies against insulin, were identical (data not shown). The FITC insulin preparations were stable for 3 months at -20 °C and could be lyophilized without loss of binding ability.

Biological potency and binding affinity of FITC-insulin

The biological potencies and binding affinities of FITC-insulin and semisynthetic human insulin are summarized in Table 1. FITC-insulin was only 51% as active as semisynthetic human insulin measured by incorporation of ^{3}H-deoxyglucose into lipid of rat adipocytes. The binding affinity of FITC-insulin was 47% of that of human semisynthetic insulin.

Effect of anti-insulin receptor antibody

U937 cells were incubated with serum from a patient with insulin receptor antibodies [21], washed 3 times in PBS, incubated in PBS for 1 h to allow dissociation from the cells of loosely bound serum factors, and finally washed once more before staining with FITC-insulin. Preincubation with receptor antibody resulted in a 55% reduction in specific fluorescence compared to cells incubated in normal serum that did not influence the FITC-insulin binding (data not shown).

Direct comparison of ^{125}I-insulin binding and FITC-insulin binding

Displacement of ^{125}I-insulin by native insulin and subsequent Scatchard analysis (data not shown) indicate that the mean number of receptors per U937 cell (R_o) is about 60,000 receptors. Estimation of the absolute num-

Table 1. Biological activity and binding of FITC-insulin and semisynthetic human insulin

Hormone	Biological activity[a]		Binding affinity[c]	
	ED_{50}[b]	relative activity %	IC_{50}[d] pM	relative affinity %
FITC-insulin		51	596	47
Semisynthetic human insulin		100	280	100

[a] Isolated rat adipocytes (2 mg lipid/ml) were incubated for 2 h at 37 °C in Krebs Ringer bicarbonate buffer (1% albumin), pH 7.4 with 0.5 μCi ^{3}H-D-Glucose and various concentrations of FITC-insulin and semisynthetic human insulin. The incorporation of ^{3}H-deoxy-glucose in lipids was measured as described elsewhere [20].
[b] ED_{50} = Concentration of FITC-insulin or unlabelled hormone that produced half maximal stimulation. [c] U937 cells (10^6/ml) and ^{125}I-insulin (20 pmol/l) were incubated with various concentrations of native and FITC-labelled insulin for 90 min at 17 °C in RPMI 1640 pH 7.4. Insulin binding was determined by centrifugation of cells through ice-cold FCS and counting of the pellet radioactivity in a γ-counter. [d] IC_{50} = Concentration of FITC-insulin or unlabelled insulin that inhibited 50% of the ^{125}I-insulin binding in U937 cells

Table 2. Estimated absolute number of insulin receptors on cultured human cells by binding of FITC-insulin or ^{125}I-insulin

Cell line	Ligand	
	FITC-insulin[a]	^{125}I-insulin[b]
IM-9	~140500	~180000
U 937	~ 40200	~ 61000
HL 60	~ 21600	~ 18200
RH-L4	~ 1000	~ 1200
DAUDI	NS[c]	NS

[a] Cells were stained with FITC-insulin (0.1 μg/ml), and the number of receptors was estimated by conversion of the fluorescence signals into absolute numbers of FITC-molecules bound per cell. [b] Cells were incubated with 20 pmol/l ^{125}I-insulin. The number of receptors was derived from Scatchard graphs. [c] NS, non-significant specific binding

ber of insulin receptors from FITC-insulin data was done by using a calibration curve, which permits conversion of mean fluorescence to absolute number of fluorescein molecules. The F/P ratio of FITC-insulin was 0.98; the number of bound insulin molecules (R_o) could therefore be calculated from the absolute number of bound FITC molecules (Table 2). The number of receptors estimated by the two methods correlated well ($r = 0.902$, $p < 0.01$).

Analysis of mononuclear leucocytes by FITC-insulin/FACS

Mononuclear blood cells comprise both lymphocytes and monocytes that can be distinguished by the scatter signal [12], because monocytes are larger than lymphocytes (Fig. 7). The FITC-insulin/FACS method showed that lymphocytes have a low amount of insulin receptors compared with monocytes, which express a significant amount of insulin receptors on the cell surface (Fig. 7). The monocyte population comprised in the
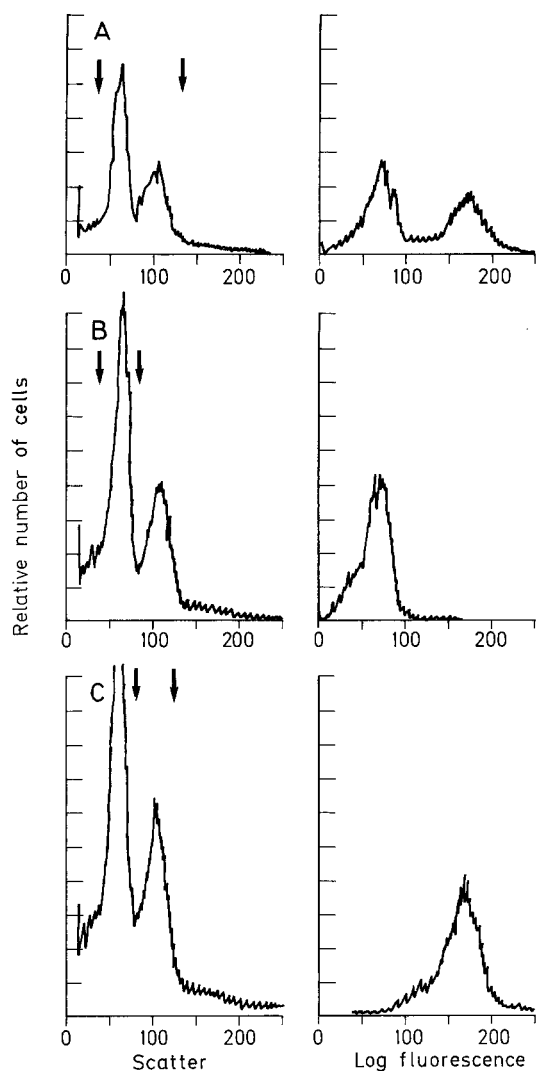
Fig. 7a–c. FACS analysis of monoclear blood leukocytes (PMNc) stained with FITC-insulin. Cells were incubated for 90 min at 17 °C with 0.1 μg/ml of FITC-insulin, washed once in ice cold PBS, fixed in 0.5% formaldehyde, and analysed by FACS. The left histograms indicate scatter signals and the right histograms are the fluorescence signals of these cells that have a scatter signal within the scatter "gate", defined by arrows. **a** total PMNc population **b** lymphocytes and **c** monocytes

shown case ~15% of the total mononuclear peripheral blood cell compartment, but may vary very significantly (from 2–20%) from individual to individual. A general average number of monocytes in blood is therefore associated with a very large standard deviation.

However, the analyses shown here indicate that cellular subsets with different amounts of insulin receptors within a cell population conveniently may be identified by the FITC-insulin/FACS method.

Discussion

The complex and heterogeneous binding kinetics of insulin to various types of eukaryotic cells seem related to differences in the amount of insulin receptors, and to differences in the affinity between insulin and the specific receptor [23]. The latter may, on the other hand, partly be a result of variations in the molecular structure of the receptor [23]. Correlations between this variability in insulin receptor expression and other phenotypic features have been difficult to establish, because the traditional methodology for studying insulin receptor expression has been based on labelling with ^{125}I-insulin that permits evaluation of the average insulin receptor expression within a cell population, rather than on the single cell level. A number of investigations have, however, clearly demonstrated that even morphologically homogeneous cell populations may be diversified with respect to a number of phenotypic features [1, 24]. It is therefore highly important that methods be developed to permit studies on insulin receptor expression on the single cell level.

The use of fluorochrome-conjugated insulin has previously been applied to investigations on insulin receptors. Most of these investigations have used FITC-insulin, but data on purification procedures and on the biological characteristics of FITC-insulin are scarce. Bromer et al. [18] reported ~50% reduction of the biological activity of FITC-insulin preparation as determined by a rat convulsion assay. This finding is in line with the reduction in biological activity of the FITC-insulin preparation used in the present study as measured by glucose incorporation into rat adipocytes. In contrast, other preparations of FITC-insulin or insulin conjugated to rhodamin and lactoalbumin-rhodamin have been reported to have only 1.15% biological activity and 8.3% binding affinity compared to native insulin [9, 10]. Several of these studies have also combined staining of cells with FITC-insulin with flow cytometry [6, 8], but none of these reports have in detail described the optimal conditions for staining with FITC-insulin. A few of these reports have provided more detailed data for the binding of FITC-conjugated lectins (in particular Con A), but the binding kinetics of Con A with its high affinity to a number of different glycoproteins cannot readily be deduced as valid for the highly specific binding of insulin to its specific receptor. Reverse phase HPLC analysis showed that FITC-conjugation resulted in different FITC-insulin molecules of which only a part could be used in FITC-insulin/FACS analysis of insulin receptors. It is also of note that the pH optimum for staining with FITC-insulin differed from the conditions for ^{125}I-insulin labelling, and it is possible that some of the former investigations with FITC-insulin have been carried out under suboptimal conditions.

The binding of the FITC-insulin preparation used in the present study was fast, saturable, reversible, and displacable by native insulin and by antibodies against insulin receptors, which all are characteristics known from ^{125}I-insulin labelling of cells. The FITC-insulin/FACS method could be used to estimate the total amount of insulin receptor (high affinity plus low affinity), but not to estimate only high affinity receptors, be-

cause labelling of only these receptors with FITC-insulin would result in a fluorescence signal of the single cell that is below the sensitivity level of the FACS. It should also be noted that the FITC-insulin/FACS method to estimate the number of insulin receptors (R_o) does not rely on a Scatchard analysis in contrast to the ^{125}I-insulin method. The two methods nevertheless resulted in comparable data for the *total* amount of insulin receptors.

The ^{125}I-insulin labelling method permits an estimation of the average number of insulin receptors per cell and can therefore not be used to measure variability in the number of insulin receptors between individual cells within a cell population. In contrast, this is feasible with the FITC-insulin/FACS method. The latter method can be used on both fixed and unfixed cells, and subsets of viable cells with different amounts of insulin receptors on the cell surface can therefore be identified and isolated by FACS. This was demonstrated by the analysis of mononuclear blood leucocytes (lymphocytes and monocytes). Monocytes had significant higher amounts of insulin receptors than lymphocytes according to the FITC-insulin/FACS analysis, which is in accordance with ^{125}I-insulin studies [25].

The FITC-insulin/FACS method should therefore permit hitherto unfeasible analysis of correlations between insulin receptor expression and other phenotypic characteristics of eukaryotic cells.

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