

**QUANTITATION OF mRNA FOR THE β -ADRENOCEPTOR GENE IN
HUMAN MONONUCLEAR LEUCOOCYTES BY IN SITU
HYBRIDIZATION WITH FLUOROCHROME LABELED CLONED DNA**

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ABSTRACT

Determination of the transcriptional activity of the β -adrenoceptor (β -AR) gene in human mononuclear leucocytes (MNL) may provide important information concerning variations in receptor expression in clinical studies. We used a new method with highly fluorochrome-labeled gene probes for in situ hybridization which allows a rapid detection of mRNA at the cellular level. MNL from venous blood of healthy volunteers were permeabilized, fixed and attached to adhesive glass slides. After incubation with fluorochrome-labeled cloned hamster β AR-DNA probes the amount of DNA-mRNA hybrids formed in the cytoplasm of individual cells was analyzed by microfluorimetry of specifically bound fluorochrome. In nine of ten normal subjects studied so far a fraction of β AR-mRNA positive MNL could be detected. The proportion of positive cells as well as their signal intensity revealed a high interindividual variance.

INTRODUCTION

Mononuclear leucocytes (MNL) from human peripheral blood contain a homogeneous population of β_2 -adrenoceptors (β -AR) (1,2). They are often employed as a tissue model to monitor variations in the expression of the β -AR-protein which can be determined

quantitatively by radioligand binding techniques (1-4). Various studies have demonstrated changes in binding characteristics of the receptor protein under physiological (5,6) and pathological conditions such as hypertensive (7-11), asthmatic (12-14) and leukemic (15,16) diseases. In this connection the question arises whether the altered receptor-expression is due to variations in the degree of gene activation and/or other mechanisms. Therefore quantification of the transcriptional product, the mRNA, may contribute important information concerning the nature of such variations at the cellular level.

Biochemical methods routinely used for investigation of mRNA, such as blot hybridization, require large and uniform cell populations for analysis and provide information only for whole populations, not for individual cells (17). In situ hybridization, in contrast, is applicable to relatively small cell populations (18) and can also yield results in heterogeneous populations such as MNL. Moreover it allows for identification and localization of the mRNA of interest in individual cells. Conventional methods using radioactively labeled gene probes are hazardous to handle and time-consuming, require special precautions, and do not provide quantitative results. Fluorochrome labeled gene probes on the other hand are easy to trace by fluorescence microscopy and their use offers the possibility of quantifying the results (19-22).

We have adapted this method to quantify the transcriptional activity of the β -AR gene in human MNL. The results from 10 normal subjects studied so far are presented.

MATERIALS

DNA probes employed in this study were derived from the expression vector for the hamster β -AR pSV β AR (complete plasmid or HIND III-fragment) (23,24) which was kindly provided by R. Dixon (Merck Sharp & Dohme Research Laboratories, West Point, Penns., USA). As control we used the plasmid pSVL alone or salmon sperm DNA (Boeringer Mannheim, Stockholm).

Hybridization buffer consisted of 10^{-2} mol/l TRIS HCl, pH 7.5, 1×10^{-3} mol/l EDTA, 6×10^{-2} mol/l NaCl, 0.02% Ficoll and 0.002% polyvinylpyrrolidone. Phosphate buffered saline solution (PBS) contained 1.4×10^{-1} mol/l NaCl, 1×10^{-2} mol/l sodium phosphate, pH 7.2; SSC buffer contained 1.5×10^{-1} mol/l NaCl and 1.5×10^{-2} mol/l sodium citrate, pH 7.5. Fluorescein isothiocyanat (FITC) was obtained from Fluka (Buchs, Switzerland), polyethyleneimine (PEI) from Serva (Heidelberg, FRG); deionized formamide and buffer substances were from Sigma chemicals or Merck (Darmstadt, FRG).

METHODS

Labeling of DNA

Fluorescein isothiocyanate (FITC) was conjugated to the synthetic polypeptide polyethyleneimine (PEI) at an FITC/PEI ratio of 9 μ g/5 mg as previously described (21,25). The degree of labeling was measured by absorbance at 280 nm for the peptide and 490 nm for fluorescein. DNA probes were denatured and then coupled to the fluoresceinated PEI using 0.04% glutaraldehyde. Excess binding capacity of the glutaraldehyde was saturated by adding 0.3% bovine serum albumine (BSA).

Cytological Preparations

Venous blood was obtained from the antecubal vein of healthy volunteers, 15-53 years old, and anticoagulated with Na_2EDTA . Immediately thereafter the MNL fraction was harvested by density gradient centrifugation (26) using Ficoll-Isopaque and washed twice in PBS. 10^6 cells were sedimented in 10 μ l of PBS. The cell membrane was made porous by suspending the cells in 500 μ l of hypotonic medium (0.9% trisodium citrate), and five minutes thereafter, the cells were fixed to retain their shape by adding 500 μ l of acetone. Fixation was allowed to proceed at least for two hours. The cells were then

attached to adhesive glass slide areas (Superior, Bad Mergetheim, FRG) at a density allowing at least 3 cell diameters of free space between individual cells.

In Situ Hybridization

Hybridization reactions were carried out with a modified method of Fournier et al. (27). 10 μ l of a dilution of the fluorochrome labeled DNA-probe in hybridization buffer (10 ng/10 cells) was added to each cell preparation on the glass slides. Reactions were sealed under coverslips using a vulcanizing glue and the slides were incubated for 48 to 72 hours in a humidified atmosphere at 37°C. The slides were then unsealed in SSC buffer, washed once in hybridization buffer containing 50% deionized formamide and once again in the hybridization buffer without formamide. They were then washed in SSC, covered with one drop of glycerine, and the fluorescence measured by microfluorimetry.

Measurements

Measurements were performed on a Leitz Orthoplan microscope photometer MPV 2 equipped with a Ploem optique for fluorescein illumination. Cells were adjusted into a measuring diaphragm under visual control and excited (480 nm) individually for 0.5 sec. Fluorescence intensity was detected by a phototube, and values were recorded, transferred to a Commodore CM 8032 and stored on a floppy disk. Relative intensity values were standardized with an external fluorescence standard. Background measurements were performed on cell-free spaces adjacent to the cells and subtracted automatically from the total cellular fluorescence value. Corrected net values were plotted as a frequency distribution diagram. Specific fluorescence intensity was calculated from the difference between the signals of β AR- and control probes. At least 100 cells were analyzed per sample.

RESULTS AND DISCUSSION

The reported method was developed and standardized in various studies on quantitation of B- and T-lymphocyte antigene-receptor mRNAs in MNL of normal donors and leukemic patients (19-22). It allows a more rapid and exact discrimination between background and specific signals than with radioactively labeled probes, especially in the low intensity range. Even minority populations of cells can be detected, since for a given total amount of mRNA, one can distinguish whether all cells express low levels or only a fraction of cells expresses high levels (19-22).

We now have applied this technique to quantify the mRNA coding for β -ARs in MNL at the individual cell level. Conditions for hybridization were optimized empirically using the maximal signal to background ratio.

Ten normal subjects have been studied so far. In 9 cases β -AR-mRNA could be detected in isolated MNL. In these cases the proportion of positive cells as well as their signal intensity (i.e. transcriptional activity) revealed a high interindividual variance. At least two subpopulations became evident: a larger population of β -AR-mRNA-negative cells (60-97%) with a fluorescence intensity distribution peak in the range of the negative control (background binding always being assayed with an unrelated fluorochrome labeled probe) and a second smaller β -AR-mRNA positive population (3-40% cells with higher fluorescence-intensities in the range of 1000 to 2000 bound probe-molecules). Four cases revealed an additional population of uncertain positive cells which was not sufficiently distinguishable from the negative population by intensity (FIG. 1). Accumulated results from all studied subjects are shown in TABLE 1.

These results demonstrate that in healthy subjects only a fraction of MNL of peripheral blood express mRNA encoding for β -ARs. Work is in progress in order to identify by double labeling with surface markers and gene probes the cell population that expresses the recep-

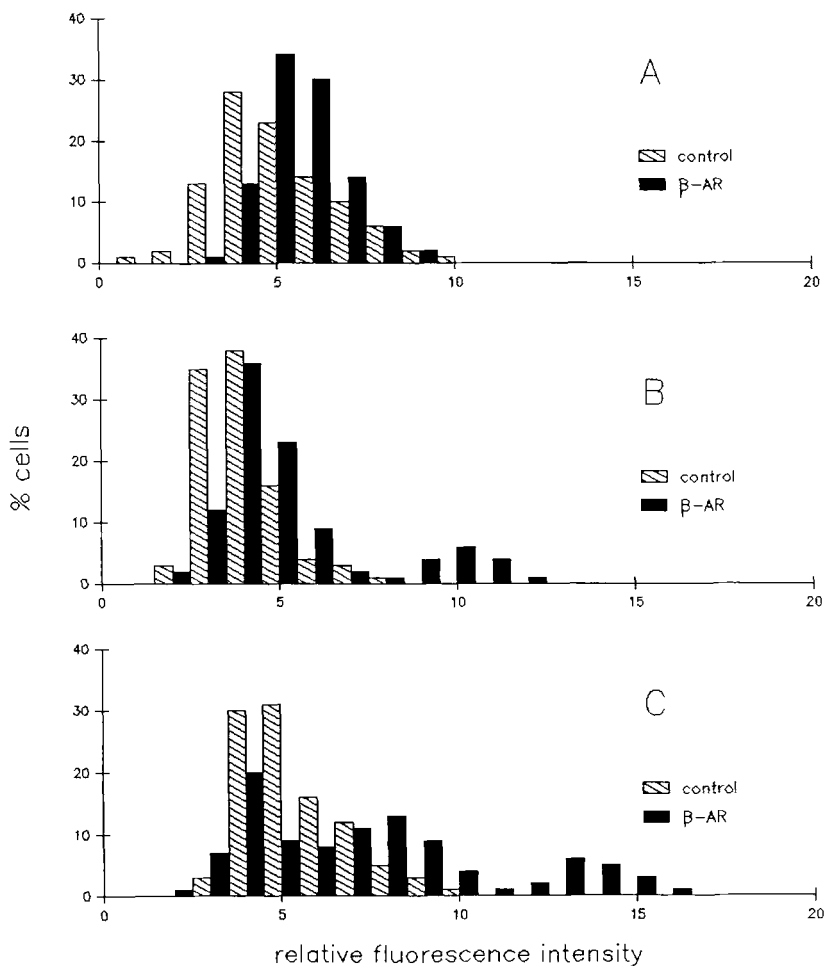


FIG. 1. Fluorescence intensity distribution of individually measured cells from 3 normal subjects hybridized with the FITC-labeled β AR-DNA fragment or salmon sperm DNA (controls) (relative fluorescence intensities related to an external fluorescence standard and corrected from background fluorescence). In panel A all cells are β AR-mRNA negative. Panel B reveals a large population of negative and a small fraction of positive cells. Panel C shows between positive and negative cells a third fraction of uncertain positive cells.

TABLE 1.
 β AR-gene Expression in MNL of 10 Healthy Subjects

subject Nr. (age, sex: f=female, m=male)	β AR-mRNA positive cells		uncertain positive cells	
	proportion [%]	mRNA-conc. at frequency peak [molec./cell]	proportion [%]	mRNA-conc. at frequency peak [molec./cell]
1 (37y.,m)	14	1500	38	500
2 (39y.,f)	38	1000	--	--
3 (53y.,f)	3	1375	--	--
4 (45y.,m)	34	1250	--	--
5 (40y.,m)	35	2000	--	--
6 (31y.,m)	--	--	31	500
7 (53y.,f)	16	1625	27	500
8 (37y.,m)	23	1250	5	250
9 (28y.,f)	15	1500	12	250
10 (15y.,m)	40	1250	--	--

tor mRNA. Further investigations will show whether the β -AR-mRNA-positive fraction of MNL is related to distinct subpopulations (e.g. B- or T-cells) and whether variations can be detected under physiological or pathological conditions.

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