New Approach to Vital Analysis of Functional Activity in ABC Transporters (Markers for Multidrug Resistance) in Solid Tumors by the Method of Flow Cytofluorometry

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We developed and described a new approach to vital analysis of functional activity of multidrug resistance markers (ABC transporters) in intact biopsy specimens from human solid tumors by the method of flow cytofluorometry. The algorithm of the study underwent revision, and the cell suspension was obtained in the final stage. Intensification of intracellular doxorubicin accumulation (fluorescence) and increase in the number of fluorescent cells and total fluorescence of doxorubicin-accumulating cells produced by ABC transporter inhibitor sodium azide served as the criteria of expression of these transporters in tumor tissue. Informative value of changes in various parameters of doxorubicin fluorescence is discussed. The increase in the count of fluorescent cells in the suspension of tumors cells after treatment with the inhibitor indicates the presence of tumor cells absolutely resistant to this preparation. The proposed method is technically simple, suitable for structurally different tumors, requires small amounts of the biopsy material and, therefore, can be used for routine analysis. The results of our analysis and spectrofluorometric assay of ABC transporters agree very closely, which suggest that this method is adequate for the purpose.

Key Words: *ABC transporters; markers for multidrug resistance; study of functional activity; human solid tumors*

The development of multidrug resistance (MDR) is associated with expression of reverse transport of antitumor preparations from cells, decrease in their intracellular concentration, and reduction of effectiveness. The development of resistance to one cytostatic is accompanied by irresponsiveness to other drugs differing in their structure and mechanism of action.

Reverse transport is performed by energy-dependent proteins belonging to the family of ABC transporters. P-glycoprotein [2,8,9], MRP [2,9], LRP [2,3, 8], and BCRP are now identified [7,11,14].

The method of flow cytofluorometry allows evaluation of functional activity of ABC transporters in the suspension of tumor cells. This technique is widely used in studies of cultured cells and various forms of leukemias. This functional test, but not assay of transport proteins or encoding genes in cells, accurately predicts the efficiency of antitumor therapy and the course of the disease and, therefore, serves as a reliable criterion for MDR [4,6,13]. Only functionally active transporters can eliminate antitumor preparations from cells and regulate their biological activity.

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The presence of transport proteins in cells or expression of encoding genes does not necessarily reflect functional activity of the transporter, which is required for its biological functions.

The ratio of solid tumors is relatively high. It was impossible to evaluate functional activity of ABC transporters in these tumors. Even gentle methods of cell isolation from solid tissue are associated with uncontrolled damage to cells and introduce appreciable errors into measurements of functional activity.

We previously elaborated a spectrofluorometric method for studying functional activity of ABC transporters in intact samples of solid tissue. This method suggests evaluation of changes in intracellular doxorubicin (DR) accumulation under the influence of specific inhibitors of reverse transport [1]. It should be emphasized that accumulation of intracellular DR in the sample is determined by a decrease in the amount of this preparation in the incubation medium, but not by its content in the tissue. This distinctive feature of the method allowed us to study intact samples of solid tumors in real time and simplified the measurements. However, the method is laborious and requires a considerable amount of material for evaluating functional activity of several ABC transporters in one tissue sample. Moreover, this method cannot be used for studies of soft tumors.

Here we adapted the method of flow cytofluorometry for studying functional activity of ABC transporters in intact tumor samples.

MATERIALS AND METHODS

Experiments were performed with biopsy specimens from various human solid tumors, including intraductal breast cancer, colon cancer, stomach cancer, and cancer of the uterine body and cervix. We examined 12 tumors. Samples were obtained at the Department of Pathological Anatomy of Tumors (N. N. Blokhin Russian Research Center for Oncology).

Functional activity of ABC transporters in tumors was additionally determined using a previously developed method of spectrofluorometry to evaluate informative value of flow cytofluorometry adapted for studying solid tumors [1].

Solid tumors were studied routinely by the method of flow cytofluorometry. Stage I suggests isolation of cell suspension by mechanical disaggregation of samples after preincubation with proteolytic enzymes. However, isolation of the cell suspension from solid tissue produces more or less pronounced damage to cells, which can introduce large errors into the measurements of functional activity of transporters. We avoided this uncontrolled effect by changing the sequence of treatment.

At stage I tumor biopsy specimens were incubated in Hanks solution with 5 mM glucose at 37°C for 30 min to restore energy processes in cells. The biopsy specimen was cut into 1-mm^3 fragments with a sharp scalpel. Each fragment was thoroughly washed with Hanks solution to remove destroyed cells. Tumor imprints for cytological assay were prepared using a surgical forceps. Samples were placed in wells A and B (Hanks solution) or C-C_n (Hanks solution and specific inhibitor of ABC transporters). Incubation was performed 37°C for 20 min. After incubation DR in a final concentration of 6×10^{-5} M was added to wells (except for control well A), and tissue samples were incubated at 37°C for 20 min.

At stage II we obtained the cell suspension for a flow cytofluorometry. Tissue samples were consecutively taken from wells B, C, and A with an anatomical forceps, put in a tube with 0.9 ml 0.9% NaCl, closed with a plug, thoroughly shaken 3-5 times depending on the density of tissues, and mixed with 0.1 ml 100% formalin (final concentration 10%). DR was added to wells at 30-sec intervals for maximum standardization of the incubation period at stage I. The cell suspension was obtained at stage II at the same time intervals.

The samples were then stored in 10% formalin until histological examination.

Immediately before cytofluorometry the cell suspension was filtered through a capron filter (pore size ~150 μ) to remove conglomerates formed during storage in a refrigerator at 4°C.

It is not accidentally that we selected this method for isolation of the cell suspension. In previous experiments the cell suspension was obtained by mechanical disintegration and centrifugation of tumor samples. Cell suspension was also obtained after 20-min rotation on a magnet stir plate. However, the count of DR-accumulating cells in the suspension was very low even after long-term incubation with high DR concentration. There were at least 2 reasons for this fact. First, DR can be removed from cells during long-term isolation of the suspension. And second, DR-containing cells in surface layers of the sample could be "diluted" by other cells localized in deeper layers and inaccessible for DR. The method for isolation of cell suspension used in our experiments complied with 3 requirements. First, the number of cells in the suspension was sufficient for flow cytofluorometery. Second, the time for cell isolation was <30 sec. And third, the time of incubation with DR and inhibitor was maximally standardized.

Sodium azide in a final concentration of 10 μM (Sigma) served as ABC transporter inhibitor. This compound is widely used as the inhibitor of energy-dependent ABC transporters to determine expression of the MDR phenotype [9,17]. DR in a final concent-

ration of 6×10^{-5} M (Pharmacia) served as a model substrate for ABC transporters.

The measurements were performed on a FACScan flow cytofluorometer (Becton Dickson) equipped with an air-cooled argon laser (488 nm) using Lysis II software. Parameters of the device were optimized using CaliBRITE™ Beads kit (Becton Dickson). The pool of cells (no less than 10,000) was accumulated in gates by means of frontal (FSC) and side light scattering (SSC) to exclude destroyed cells and cellular conglomerates from the study. Isolation and analysis of cells were performed by FL-2 parameter. Fluorescence (FL) signals of intracellular DR were recorded at 585 nm. Histograms were analyzed by mean FL of cells, number of FL-cells, and total FL of cells in the suspension.

RESULTS

It should be emphasized that the proposed approach to studying solid tumors by the method of flow cytofluorometry allowed evaluation of intracellular DR accumulation. FL of cells in the suspension was sufficient for quantitative analysis of various parameters. We revealed differences in intracellular DR accumulation not only in various human solid tumors, but also in tumors with the same localization and histological composition. Fig. 1 shows distribution histograms of cells from intraductal breast cancer depending on the intensity of FL. The zone of FL not overlapping with FL of control cells incubated in Hanks solution included 1 (Fig. 1, *4*) or 2 dominant peaks (Fig. 1, *1-3*).

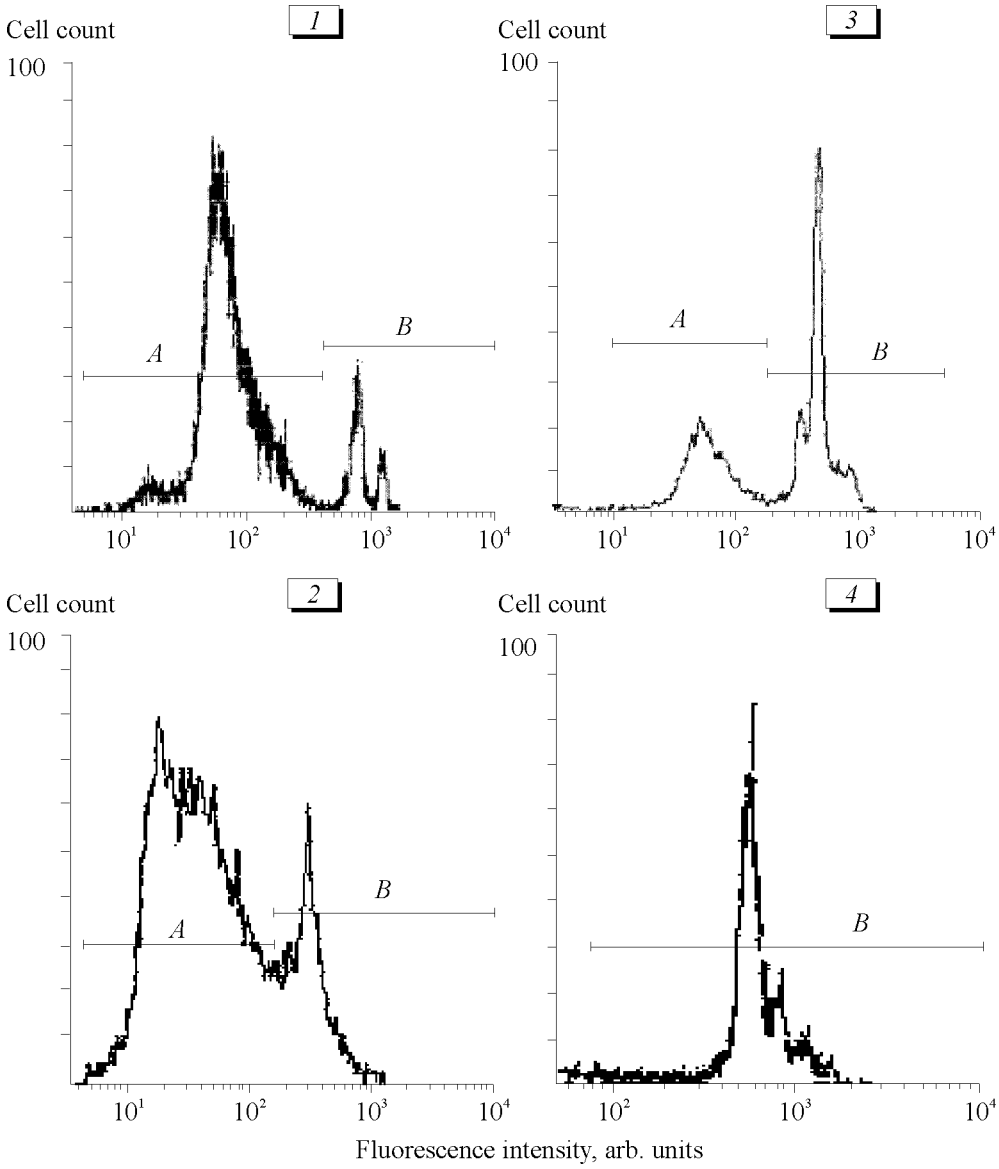

Fig. 1. Histograms of fluorescence for breast cancer cells from various patients after incubation with doxorubicin (*1-4*). Here and in Fig. 2: zones of low (*A*) and high fluorescent cells (*B*).

TABLE 1. Intracellular DR Accumulation in Cell Suspension with Single FL Peak

Treatment	Absolute number of FL-cells accumulating DR (%)	Mean DR FL	Total FL of cells accumulating DR ($\times 10^3$)	Increase in mean FL of suspension	Tumor phenotype	
					FCF	SF
Breast cancer, patient B.						
DR	4372 (44%)	206	900	90	MDR^-	MDR^-
Sodium azide and DR	4555 (45%)	178	810	81		
Sodium azide and DR / DR	1.0	0.9	0.9	0.9		
Cancer of the uterine cervix, patient N.						
DR	2014 (20%)	106	213	18	MDR^+	MDR^+
Sodium azide and DR	2978 (30%)	196	584	40		
Sodium azide and DR / DR	1.5	1.8	2.7	2.2		
Cancer of the stomach, patient I.						
DR	2060 (21%)	329	677	80	MDR^+	MDR^+
Sodium azide and DR	3263 (32%)	406	1325	144		
Sodium azide and DR / DR	1.5	1.2	2.0	1.8		
Breast cancer, patient G.						
DR	2561 (26%)	116	297	32	MDR^+	MDR^+
Sodium azide and DR	5167 (52%)	92	475	49		
Sodium azide and DR / DR	2.0	0.8	1.6	1.5		
Cancer of the uterine body, patient R.						
DR	4935 (49%)	110	543	51	MDR^+	MDR^+
Sodium azide and DR	4898 (49%)	250	1225	122		
Sodium azide and DR / DR	1.0	2.3	2.3	2.4		

Note. Here and in Table 2: FCF, flow cytofluorometry; SF, spectrophotometry.

Tumors differed in the intensity of FL in various regions and the number of cells in each fraction. The number of cells was maximum in the zone of low (Fig. 1, *1*, *2*, *A*) or high FL (Fig. 1, *3*, *B*). Minor peaks were revealed in zones of high or low FL.

Heterogeneity of cells from the same tumor by the intensity of DR FL and differences in the distribution of low- and high-intensity peaks of FL in various tumors illustrate the diversity of solid tumors by several parameters. At least 2 factors can determine intracellular DR content. First, tumors are heterogeneous by their density. Previous studies showed that more than 90% DR bind to cell DNA. Therefore, this fraction of anthracycline determines the intensity of FL in cells. Cells with higher DNA content (*i.e.,* greater ploidy) would be localized in the region of intensive FL, all other factors being equal. And second, intracellular DR content is determined by its elimination from cells associated with functional activity of ABC transporters. The phenotypic distribution of ABC transporters can differ in various cells of the same tumor. Only simultaneous measurements of FL in the cell population, its ploidy, and functional activity of ABC transporter would allow evaluating the major characteristic determining DR FL in the heterogeneous population of tumor cells. This assay is methodically simple, since ploidy of samples from the control suspension can be determined by staining with propidium iodide [12]. It was the next goal of our study. In the present work tumors were characterized by expression of functional activity of ABC transporters determining the MDR phenotype.

We compared the type and intensity of DR FL in cells of intact samples and tumors treated with ABC transporter inhibitor sodium azide. Sodium azide is widely used for this purpose, since the inhibition of

energy processes in cells is followed by suppression of expressed ABC transporters with energy-dependent functional activity [5,10]. Flow cytofluorometry was performed in parallel with spectrofluorometric assay of functional activity of ABC transporters from tumor biopsy specimens. Similar conclusions were drawn regarding the MDR phenotype.

Table 1 shows intracellular DR accumulation in the suspension of tumor cells with 1 dominant zone of FL. In sample 1 (breast cancer, patient B.) the number of FL-cells, mean FL, total FL of cells accumulating DR, and increase in total FL of cells incubated with DR remained unchanged after treatment with the inhibitor. These data suggest that sodium azide had no effect on intracellular DR accumulation. Therefore, the test cells contained no functionally active ABC transporters (MDR^- tumor phenotype).

In other samples sodium azide increased mean FL and total FL of cells accumulating DR by 1.5-2.5 and 1.6-2.7 times, respectively. These parameters characterize total accumulation of DR in tumors that was intensified after treatment with sodium azide. These data suggest the existence of cells with functionally active ABC transporters. The inhibition of ABC transporters induced intracellular DR accumulation and increased FL of the cell population.

In 3 of 4 tumors (cancer of the uterine cervix, cancer of the stomach, and breast cancer) the number of FL-cell increased by 1.5-2.0 times. Treatment with sodium azide 1.8-fold increased mean FL of DR-accumulating cells only in the sample from cancer of the uterine cervix.

In the sample from cancer of the uterine body the number of FL-cells accumulating DR remained unchanged; other test parameters, including mean DR FL, increased by 2.5 times.

Changes in various parameters of FL produced by sodium azide indicate that test tumors express functional activity of ABC transporters (MDR^+ phenotype).

Table 2 shows tumors containing fractions of cells with low (A) and high FL (B) after incubation with DR. We evaluated individual reactions of cells to sodium azide in 2 fractions with various parameters of FL.

Studies of 2 breast tumors showed that sodium azide does not affect FL in fractions of cells with low

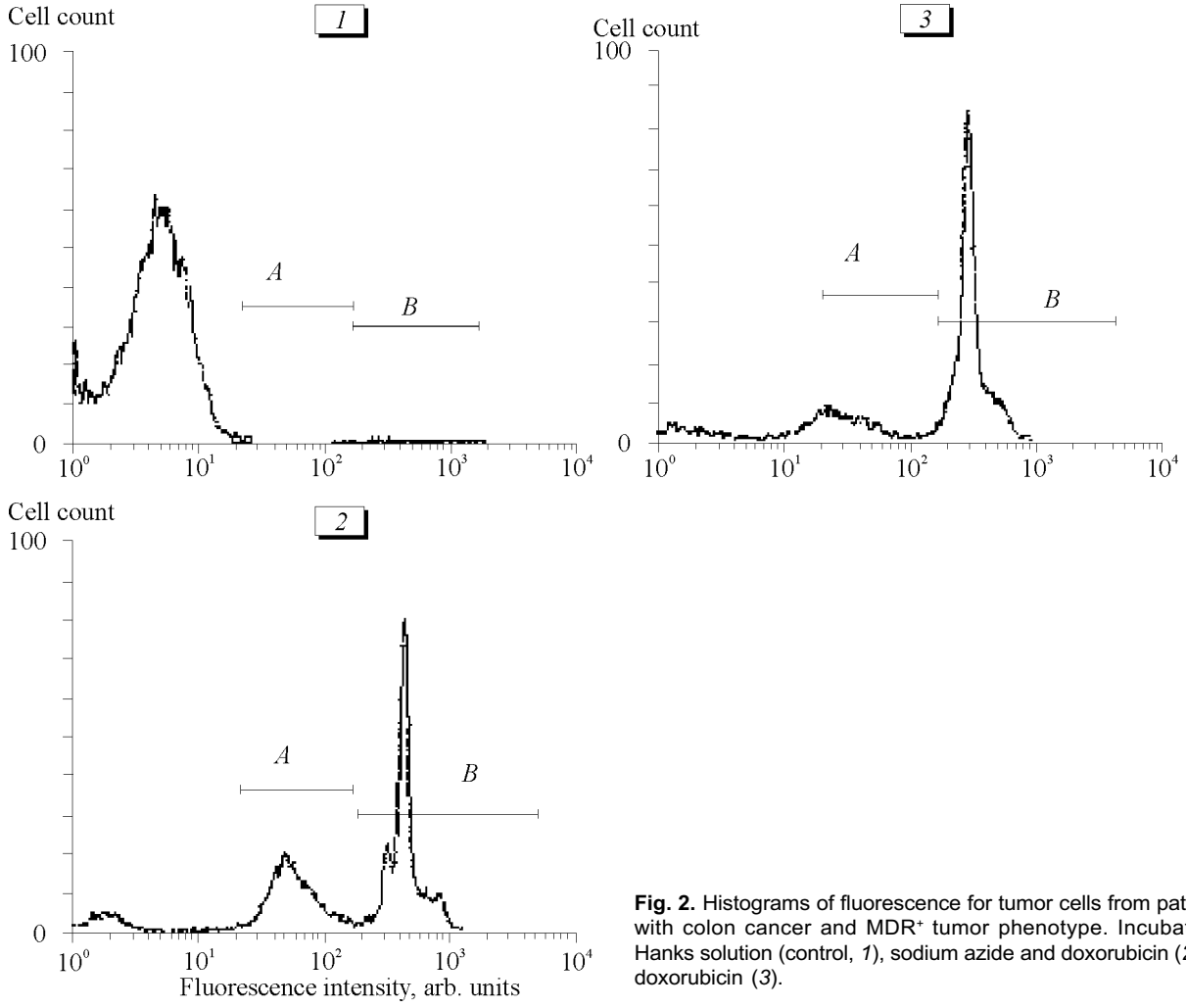

Fig. 2. Histograms of fluorescence for tumor cells from patient S. with colon cancer and MDR^+ tumor phenotype. Incubation in Hanks solution (control, *1*), sodium azide and doxorubicin (*2*), and doxorubicin (*3*).

TABLE 2. Intracellular DR Accumulation in Cell Suspension with Two Peaks of FL

Cell fraction*	Treatment	Absolute number of FL-cells accumulating DR (%)	Mean DR FL	Total FL of cells accumulating DR ($\times 10^3$)	Tumor phenotype	
					FCF	SF
Breast cancer, patient A.						
A	DR	8324 (83%)	10	83	MDR^-	MDR^-
	Sodium azide and DR	8016 (80%)	11	88		
	Sodium azide and DR / DR	1.0	1.1	1.1		
B	DR	1684 (17%)	167	281		
	Sodium azide and DR	1939 (19%)	145	281		
	Sodium azide and DR / DR	1.1	0.9	1.0		
Breast cancer, patient M.						
A	DR	8813 (88%)	83	737	MDR^-	MDR^-
	Sodium azide and DR	8859 (89%)	89	788		
	Sodium azide and DR / DR	1.0	1.1	1.1		
B	DR	1186 (12%)	964	1143		
	Sodium azide and DR	1141 (11%)	931	1062		
	Sodium azide and DR / DR	0.9	1.0	0.9		
Colon cancer, patient S.						
A	DR	2092 (21%)	32	66	MDR^+	MDR^+
	Sodium azide and DR	3619 (36%)	65	235		
	Sodium azide and DR / DR	1.7	2.0	3.6		
B	DR	5442 (55%)	321	1747		
	Sodium azide and DR	6435 (64%)	480	3137		
	Sodium azide and DR / DR	1.2	1.5	1.8		

Note. Fractions of highly (A) and low fluorescent cells (B).

and high FL. The number of FL-cells, mean FL, and total FL of cells accumulating DR remained unchanged. These data indicate that sodium azide has no effect on intracellular DR accumulation. Therefore, the test cells contain no functionally active ABC transporters (MDR^- tumor phenotype).

Other results were obtained in experiments with colon cancer. Sodium azide produced most pronounced changes in the fraction of cells with low FL (A). The number of FL-cells increased, mean FL, and total FL of cells accumulating DR increased by 1.7, 2.0, and 3.6 times, respectively. Other changes were observed in the fraction of cells with high FL (B). Sodium azide practically did not change the number of FL-cells (1.2-fold increase), but increased mean FL and total FL of cells (by 1.5 and 1.8 times, respectively). Sodium azide modified various parameters of FL, which indicates that colon cancer express functional activity of ABC transporters (MDR^+ phenotype). To illustrate the reaction of tumor cells to sodium azide we constructed histograms for the distribution of cells by FL before and after treatment with the inhibitor (Fig. 2).

When histograms include 2 zones of DR FL with different intensity, activity of ABC transporters can be evaluated by comparing total FL of cells in both fractions before and after treatment with sodium azide. Similar conclusions were made regarding the MDR phenotype of the studied tumors (Table 2). Differential analysis of various parameters that can be leveled during

complex study provides additional information about the MDR phenotype of the tumors. In colon cancer the number of FL-cells after treatment with sodium azide increased only in the fraction with low FL (Table 2).

This parameter is clinically important, since it reflects the presence of tumor cells with high activity of ABC transporters not accumulating DR before treatment with sodium azide. Some tumor cells do not react to the toxic effect of MDR-preparations independently on changes in other parameters, including proliferative activity, intensity of apoptosis, and reparation. These parameters can improve or reduce the effectiveness of MDR-preparations under various MDR phenotypes. Expression of MDR is one of the mechanisms determining inefficiency of chemotherapy. The population of cells not accumulating DR is not absolutely resistant. The more pronounced is the increase in FL-cell count after treatment with sodium azide, the higher is the number of cells not accumulating DR ("absolute" resistant).

Total FL of cells accumulating DR seems to be informative. The increase in this parameter after treatment with sodium azide reflects expression of the MDR phenotype in tumors and is associated with activity of ABC transporters (*i.e.*, MDR phenotype). We believe that total FL of cells gives information important in the practical aspect. Of two tumors with the MDR phenotype, the tumor with high total FL of cells and less pronounced increase in this parameter after treatment with sodium azide has a more favorable prognosis in relation to the development of resistance.

Detailed study of changes in various parameters of FL after treatment with inhibitor(s) of ABC transporters illustrates not only expression of MDR, but also its phenotypic characteristics in various tumors.

The method of flow cytofluorometry with minor modifications (change in the sequence of stages) allows us to evaluate functional activity of ABC transporters, determine expression of MDR and intensity of this process, and reveal tumor cells "absolutely resistant" to MDR preparations in intact samples of human solid tumors. After incubation with DR the distribution of cells from human solid tumors with various parameters of FL differs from that observed in experiments with the suspension of cultured cells. This fact is not surprising. Only new approaches to studies of tumor structures would elucidate various clinical problems. The proposed method and complex approach to evaluation of the results of flow cytofluorometry seem to be successful. This method for detection of the MDR marker in intact solid tumors (functional activity of ABC transporters) is technically simple, suitable for tumors with different composition, requires small amounts of the biopsy material and, therefore, is useful for routine applications. The results of our analysis and spectrofluorometric assay of ABC transporters agree very closely, which indicates that this method is well suited for the purpose. Undoubtedly, information about expression of the MDR phenotype in tumors is important for physicians. The use of specific transporter inhibitors, simultaneous study of tumor ploidy, and identification of tumor cells by specific markers (antigens) would provide a total description of tumors and MDR phenotype.

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