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SIMULTANEOUS FLOW CYTOFLUOROMETRIC ANALYSIS OF THE CELL CYCLE AND OF SUBPOPULATIONS OF IMMUNOCOMPETENT CELLS IN WORKERS CLEANING UP AFTER THE CHERNOBYL' ATOMIC POWER STATION DISASTER

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UDC 616.001.26-06:612.017.1:576.3

KEY WORDS: immunocompetent cells; irradiation; cell cycle; cytofluorometry.

The study of the cell cycle of subpopulations of immunocompetent cells is both of direct scientific importance as a means of shedding light on the mechanisms of development of immunopathological states and also of practical importance in the search for agents deblocking the cycle and other measures of immunomodulating therapy, exerting a selective action on cells depending on their surface phenotype. A practical solution to these problems became possible as a result of sorting of cells labeled with monoclonal antibodies (MCAB) on a flow cytofluorometer, followed by culture and determination of incorporation of ^{3}H-thymidine and ^{3}H-uridine [8]. Besides the large number of stages involved, with this approach it is almost impossible to prevent the additional activating action of MCAB on peripheral blood mononuclears (PBM). An essential simplification of the technique used in this approach was to use 5-bromodeoxy-uridine (BUdR), followed by conjugation with anti-BUdR MCAB in the indirect immunofluorescence test and with MCAB to surface receptors in the direct test [7]. Recording was carried out on a single-beam cytofluorometer on two-color fluorometry mode. The use of a simple technique of staining cell DNA with the dye Hoechst 3342 in combination with MCAB to differential antigens requires dual-beam cytofluorometry [6]. In recent years the use of MCAB to surface antigens in combination with intercalating dyes for double-stranded DNA (propidium iodide or ethidium bromide), which enables changes to be recorded in the cell cycle in subpopulations of PBM, stimulated by phytohemagglutinin and concanavalin A [5], has been considered the most promising approach. To improve the staining, additional treatment was given with 0.1% saponin solution, or the material is fixed in 2% paraformaldehyde in 70° ethanol [9].

The aim of this investigation was to study the possibility of analyzing the cell cycle of unstimulated PBM from persons exposed to ionizing radiation in direct and indirect immunofluorescence tests.

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Laboratory of Clinical Immunology, All-Union Radiation Medicine Research Center, Academy of Medical Sciences of the USSR, Moscow. Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 112, No. 10, pp. 400-402, October, 1991. Original article submitted January 24 1991.

0007-4888/91/0010-1461$12.50

TABLE 1. Cell Cycle of Subpopulations of Immunocompetent Cells during Two-Color Flow Cytofluorometry with MCAB and Staining of DNA with Propidium Iodide

Cell subpopulation tested	Group of observations	Parameters of cell cycle		
		G_1	S	G_2+M
Leu1$^+$	C	90,1±4,1	5,9±0,5	5,1±0,4
	E	95,4±3,9	2,7±0,1*	1,1±0,1*
Leu7$^+$	C	89,7±3,3	8,9±0,2	5,0±0,1
	E	78,4±3,6*	11,8±0,4*	8,5±0,4*
Transferrin receptor$^+$	C	23,7±2,7	35,5±1,9	40,8±2,2
	E	26,7±1,9	31,8±1,7	40,3±1,9
Surface Ig$^+$	C	75,8±3,3	16,8±0,8	5,9±0,2
	E	70,2±5,5	18,1±1,3	10,9±1,0*
PBM as a whole	C	92,5±3,2	5,2±0,1	3,8±0,2
	E	86,3±10,8	7,2±0,8*	8,5±0,2*

Legend. Here and in Table 2: C) control group, E) experimental, Asterisk indicates significant differences between C and E groups, $p < 0.05$.

EXPERIMENTAL METHOD

The subjects studied were 35 persons exposed to predominantly external irradiation in doses of 0.05-0.25 Gy as a result of the disaster at the Chernobyl' Atomic Power Station (experimental group) and 12 persons with absorbed doses within the limits of background levels (control group), PBM were isolated in a Ficoll–Verografin density gradient [4] from 10 ml of heparinized venous blood, The cells (10^5) were incubated with 10 μl of anti-Leu1, anti-Leu7 MCAB, against the transferrin receptor ("Becton Dickinson," USA) or antibodies to light and heavy chains of human IgA + IgG + IgM ("Beringwerke," West Germany), conjugated with fluorescein isothiocyanate (FITC) at 4°C for 30 min. The cells were washed 3 times with buffered physiological saline (pH 7.2-7.4) and fixed by the addition of 5 μl of 2% formaldehyde in buffered physiological saline, after which they were stained with propidium iodide ("Sigma," USA) in a working concentration of 50 μg/ml. The specificity of DNA staining was verified by preliminary treatment with DNase ("Sigma," USA). Slow cytofluometric analysis was carried out on a "FACStar Plus" ("Becton Dickinson," USA), with wavelength of the argon laser 488 nm. The lymphocyte population was determined from values of forward (5°) and sideways (90°) scattering of light (FSC and SSC respectively). The samples were tested on two-color fluorescence mode with coefficients of compensation of 0.8% for the FL1 detector and 21.2% for the FL2 detector, with logarithmic intensification in the FL1 channel and linear in FL2. The amplification factor was chosen so that the G_1 peak of the cells was within the interval of 240-480 channels when working with the "FACStar Plus" program or 60-120 channels when working with the "Consort 30" program, For each MCAB no fewer than 5000 cells were recorded on "dot-plot" mode, limiting the cell fraction studied only to FL1 positive relative to the total population. Automated analysis of the cell cycle was carried out for populations segregated in accordance with the "Paint-a-Gate" program, using the DNA program and a polynomial model [3]. Comparative analysis of the curves was carried out by the Kolmogorov–Smirnov method, in accordance with the program provided by the manufacturer.

EXPERIMENTAL RESULTS

Data reflecting the cell cycle of the subpopulations of immunocompetent cells of irradiated individuals compared with the control are given in Table 1.

Table 1 shows that a heterogeneous peripheral blood lymphocyte population is in different phases of the cell cycle, with significant differences between control and experimental groups both for cells with Leu1, Leu7, sIg surface markers and for unfrationated PBM as a whole.

Threshold values of activation and suppression for the different lymphocyte populations and exposure to small doses of radiation differ [1]. Values of coefficients of variation of the G_1 peak (Table 2) were smaller for the subpopulations analyzed separately than for the PBM suspension as a whole, additional evidence in support of differences in the cell cycle. The ratio between mean values of the G_2 + M and G_1 peaks for the subpopulations studied also varied, although it did not go outside the limits of permissible fluctuations.

TABLE 2. Statistical Analysis of Histogram Peaks during Investigation of Cell Cycle of Lymphocyte Subpopulations

Cell subpopulation tested	Group of observation	Region of distribution	Mean value of G_1 phase (channel)	Coefficient of variation G_1	Ratio G_2+M/G_1
Leu 1^+	C	200—1023	232,4	8,3	2,0
	E	180—1000	200,1	12,7	1,56
Leu 7^+	C	268—1023	300,6	14,1	1,88
	E	300—1020	370,9	20,5	1,86
Transferrin receptor$^+$	C	240—1023	280,0	11,3	2,12
	E	256—1023	296,0	12,9	2,15
Surface Ig^+	C	324—1023	280,1	10,2	1,78
	E	360—1023	330,3	17,5	1,83
PBM as a whole	C	240—1023	290,3	16,3	1,98
	E	220—1023	270,6	27,4	1,67

The accuracy of quantitative analysis depends both on the fixation mode and on the number of cells analyzed. Initially we stained with propidium iodide after incubation of the unfixed cells with MCAB and rinsing. Under these conditions 20-27% of cells were found to have unstained nuclei, and the coefficients of variation of the G_1 peak varied between 17.3 and 43%. Later, fixation of the cells by addition of 50 μl formol-acetone (pH 6.8-7.2) or 1% paraform to the wells of the planchet was used, as is recommended for work with MCAB of the Leu series. Better results are obtained by preliminary treatment of the fixed cells with a 0.5% solution of Triton X-100 for 5 min followed by staining. While not significantly affecting the number of cells and intensity of fluorescence of the antigen, fixation facilitates differentiation of lymphocytes from macrophages and granulocytes, and also enables serial delivery of samples from 96-well planchets through an automatic sampler.

Automated analysis of the cell cycle according to the "DNA" program envisages a study of 5000 cells. Experience shows that to obtain satisfactory results with qualitative staining of the cells, it is sufficient to analyze 3000-3500 events. In practice, it is necessary to assess 10,000-20,000 cells for this purpose, when analyzing the cell cycle of $Leu1^+$, $Leu7^+$, IPO-10^+, Ia^+, and sIg^+ subpopulations. So far as cells numbering 1-5% in the peripheral blood are concerned, for analysis 60,000-100,000 PBM must be examined and the memory must have the capacity to store not more than 50,000 events on four-parameter mode, in "FACStar Plus" and "Consort 30" programs. In such cases it is advantageous to record data on two-parameter mode, and to store the information separately for lymphocyte and macrophage populations, discriminated if the results are classified according to FSC and SSC, and recorded in separate files in terms of parameters FL1 and FL2. If there are insufficient cells, the analysis is done manually [2].

Thus two-color cytofluorometry with MCAB and propidium iodide provides a rapid and reproducible method of obtaining information about the cell cycle of PBM subpopulations on manual or automatic mode. Changes in the cell cycle observed in individuals exposed to ionizing radiation call for further study.

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