Experimental Cell Research **249,** 46–53 (1999)
Article ID excr.1999.4434, available online at http://www.idealibrary.com on IDEAL®

DNA Global Hypomethylation in EBV-Transformed Interphase Nuclei

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In tumors, DNA is often globally hypomethylated compared to DNA extracted from normal tissues. This observation is usually made after extraction and exhaustive digestion of DNA followed by analysis of nucleosides by chromatography or digestion with restriction enzymes, gel analysis, and hybridization. This approach provides an average value which does not give information on the various cell subpopulations included in heterogeneous samples. Therefore an immunochemical technique was set up with the aim of demonstrating, in a population of mixed cells, the possibility of detecting the presence of individual nuclei containing hypomethylated DNA, on a cell-by-cell basis. Monoclonal antibodies to 5-methylcytidine were used to label cells grown *in vitro*. Under appropriate fixation and permeabilization conditions, interphase nuclei were labeled. Quantitative differences in the labeling were detected between Epstein–Barr virus-transformed cells and normal peripheral blood monocytes by flow cytometry analysis. Similar differences were observed by fluorescence microscopy. Both results were confirmed by Southern transfer and hybridization of DNA fragments generated by restriction enzyme digestion. This observation, which is in accordance with the occurrence of global DNA hypomethylation in tumors as established by chromatography, opens the field for the analysis of fresh tumor samples by flow cytometry and microscopy.

Key Words: **5-methyldeoxycytidine; flow cytometry; fluorescence microscopy; Epstein–Barr virus.**

INTRODUCTION

DNA methylation results from the transfer of a methyl group from *S*-adenosylmethionine to the carbon 5 of cytosine in CpG dinucleotides [1] by DNA methyltransferase, an enzyme displaying a strong affinity for hemimethylated DNA, ensuring the maintenance of the methylation pattern after DNA replication. The presence of methylated cytosines at or near the 5′ end of numerous genes has been observed in tissues in which these genes are silent. On the contrary the promoters of expressed genes are often associated with unmethylated CpGs [2, 3]. The detection of a global hypomethylation coexisting with local hypermethylated sites in DNA extracted from tumors led to the concept of an uncontrolled expression of oncogenes with a concomitant silencing of tumor suppressor genes in cancer [4, 5]. The first evidence for a genome-wide hypomethylation in cancer was brought about by analyzing DNA extracted from tumor tissues by chromatography [6, 7]. The degree of methylation of specific genes is usually investigated with restriction enzymes and molecular hybridization or genomic sequencing [8]. These techniques were successfully applied to the study of various malignancies and allowed the establishment of the fact that alterations of the methylation pattern of genomic DNA or of specific genes were an early event in the development of colon cancer [9, 10]. Similar observations were also reported for numerous other malignancies such as breast [11], prostate [12], and lymphoproliferative disorders [13].

The results quoted above were obtained after extraction of DNA and no comparison could be made between individual cells containing normally or hypomethylated DNA since the tissue or cellular architecture was destroyed during the extraction process. The possibility of using immunochemical techniques to detect the presence of 5-methylcytidine (5-MeCyd) *in situ* was initially exploited with polyclonal sera [14] to illustrate the distribution of methyl-rich regions in metaphase chromosomes, then with monoclonal antibodies [15–18]. On such samples the presence of labeled interphase nuclei was obvious and their methylation level could be quantified [19, 20]. However, observation of individual cells by nonautomated techniques is time consuming and the detection of a malignant subpopulation reaching 5% of the total population can require

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0014-4827/99 $30.00

the screening and analysis of more than 1000 cells. This observation prompted us to develop a technique aimed at the detection of putative global differences between populations of normal and transformed cells by flow cytometry. The biparametric flow cytometric study (5-MeCyd-FITC/DNA-PI) was performed with an Epstein–Barr virus-transformed cell line growing in suspension and peripheral blood cells (PBC). The effect of 5-azacytidine, an inhibitor of DNA methylation, was also investigated. Results obtained were confirmed by visual observation with the fluorescence microscope and by molecular hybridization experiments. They indicate that

(i) the presence of cells harboring a globally hypomethylated genome can be easily and rapidly detected in a mixed population,

(ii) the percentage of hypomethylation can be measured, and

(iii) the methylation status of resting and proliferating cells can be compared and measured.

MATERIALS AND METHODS

Monoclonal antibodies directed against 5-methylcytidine were obtained as previously described [21]. Their specificity toward the methyl group on carbon 5 of the pyrimidine ring has been demonstrated by inhibition experiments in which no binding occurred in the presence of the competing methylated hapten, whereas cytosine did not inhibit the reaction. This competition was observed for ELISA tests [21] as well as for labeling of metaphase chromosomes [15] and of interphase nuclei [19] and for immunoblots with DNA immobilized on nitrocellulose membranes [22].

The cell line used in this work was P3HR1 (an Epstein–Barr (EBV) virus-transformed lymphoblastoid cell line). P3HR1 cells grow in suspension and were cultivated in RPMI 1640 medium (Life Technologies, Cergy Pontoise, France) supplemented with L-glutamine, 10% newborn calf serum, and antibiotics in a 5% CO_2 humid atmosphere.

PBC were isolated from heparin-treated blood, collected from healthy volunteers by venous puncture, by centrifugation on Ficoll gradients (Eurobio, Les Ulis, France).

Hypomethylation of DNA in P3HR1 cells was induced with 3 μM freshly dissolved 5-azacytidine for 24 or 72 h. For chase experiments, cells were washed by three successive pelleting and resuspension steps at low speed in RPMI 1640, then maintained for 48 h in fresh culture medium.

Cells growing in suspension and PBC were submitted to successive low-speed (300*g*) pelleting and gentle resuspension steps in order to minimize clumping before analysis by flow cytometry. They were washed twice with pH 7.4 PBS supplemented with 1% BSA and 0.1% Tween 20 (PBST-BSA) and then were fixed with 0.25% paraformaldehyde in PBS for 10 min at 37°C. Specimens were then cooled and maintained at 4°C for 10 min before addition of 9 vol of methanol/PBS (88% methanol/12% PBS vol/vol) refrigerated at −20°C. After two washes with PBST-BSA at room temperature cells were treated successively with 2 N HCl at 37°C for increasing periods of time, then with 0.1 M borate buffer pH 8.5 for 5 min. Although acidic treatments are likely to destroy cytoplasm and hydrolyze tRNA in which 5-MeCyd can be found and recognized by antibodies (unpublished blotting results), samples were treated with RNase (10 μg/ml) for 30 min at 37°C and then were washed twice with PBST-BSA. Control experiments performed without RNase gave identical results. Then cells were treated for 20 min at 37°C with a blocking solution made of PBST-BSA supplemented with 10% newborn calf serum, before incubation with anti-5-MeCyd antibodies (hybridoma supernatant, containing 5 μg/ml antibodies). Specimens were then successively rinsed three times with PBS and incubated for 45 min at 37°C with goat anti-mouse immunoglobulins conjugated to fluorescein isothiocyanate (Dako, Trappes, France) diluted 1/50 in PBST-BSA. Finally samples were washed three times with PBS and either were mounted in phosphate-buffered glycerol for microscopy or were stained with propidium iodide (PI) (50 μg/ml in PBS) for 30 min before flow cytometry.

Analyses were performed by flow cytometry (FACScan equipped with Lysis II software; Becton–Dickinson, Grenoble, France). The low flow rate setting was used. A 560-nm dichroic mirror and a 600-nm band pass filter were used for red fluorescence. Spectral compensation was made for PI emission entering the FL2 channel. Cells were excited at 488 nm with a 15-mW argon laser and the green fluorescence emission was detected at 530 ± 15 nm. Forward light scattering was used to gate the cell subpopulations. Nonspecific antibody binding was monitored with a nonimmune mouse IgG1 isotype. DNA content was analyzed using the Cellfit software (Becton–Dickinson). The bivariate IP/FITC distribution was displayed in dot plots by counting 10^5 cells.

Relative overall DNA methylation was also determined for both cell types as previously described [23]. Briefly a constant quantity of DNA (2 μg) was digested overnight with *Msp*I (methylation insensitive) or *Hpa*II (methylation sensitive) restriction enzymes (10 units/μg of DNA), separated on 1% agarose gels, and blotted onto Hybond N membranes (Amersham). Hybridization was performed with 100 ng of random-primed, ^{32}P-labeled human placenta DNA, at 65°C in 5× SSC, 5× Denhardt's, 0.1% SDS, and 100 ng/ml denatured salmon sperm DNA. The amount of hybridized radiolabeled probe was measured with a PhosphorImager (Molecular Dynamics). The methylation status of each sample was established by measuring the ratio between the radioactivity present in the zone encompassed by 1.8- and 2.9-kb molecular weight markers and the totality of the lane. This value, multiplied by 100, was taken as an index of demethylation. It ranges theoretically from 0 (the most methylated) to 100 (the least methylated).

RESULTS

Results obtained when our anti-5-MeCyd antibodies were used to label metaphase chromosomes are illustrated in Fig. 1a. In this picture one can observe, near the metaphase spread, an interphase nucleus in which fluorescent spots can be seen. This image is currently observed after the usual methanol–acetic acid fixation procedure developed for cytogenetic studies during which a hypotonic shock is used to disrupt the nuclear membrane which also leads to the destruction of the cytoplasm. The absence of labeling with unrelated immunoglobulins established the specificity of this immunolabeling as previously published [15].

For cells growing in suspension immunolabeling could be achieved after treatment with 2 N HCl at 37°C. Differences could be consistently detected between unstimulated (Fig. 1b) and stimulated human PBC (Fig. 1c) and continuously growing P3HR1 cells (Fig. 1d), with a clear progressive decrease of the fluorescence from the former to the latter. These preliminary experiments were performed with a small num-

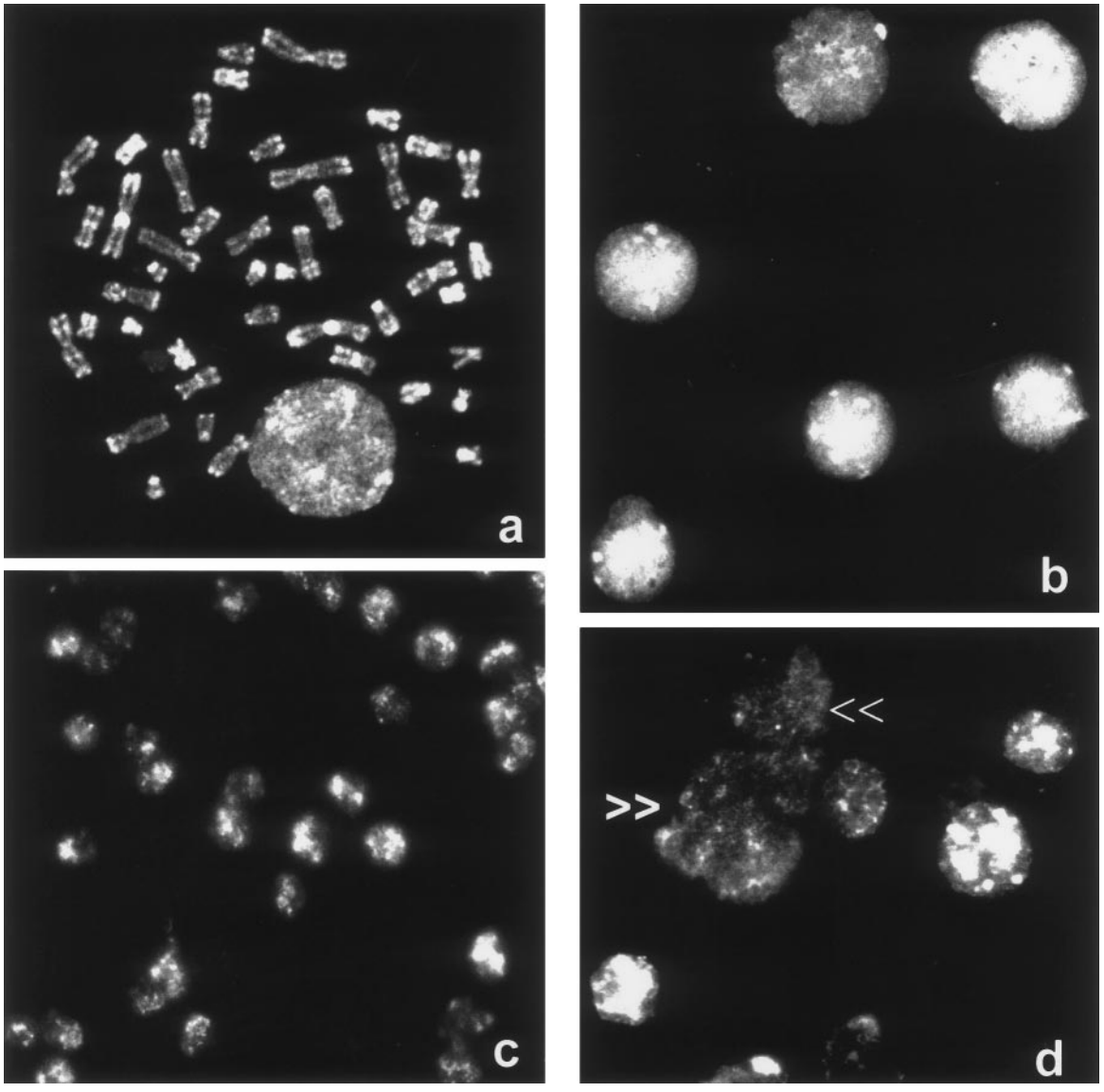

FIG. 1. Labeling of chromatin structures with anti-5-MeCyd antibodies. (a) Metaphase chromosomes were obtained with the classical hypotonic shock–UV irradiation method. An interphase nucleus is seen in which the chromatin is labeled. (b) Peripheral blood cells unstimulated, treated, and labeled as described under Materials and Methods. (c) Peripheral blood cells stimulated with PHA and treated as above. (d) A mixture of unstimulated peripheral blood cells and P3HR1 cells (double arrows).

ber of cells. Therefore larger populations were investigated by flow cytometry.

In order to obtain the best compromise between cell integrity and fluorescence intensity, the duration of acidic treatment was varied between 0 and 180 min. As shown in Fig. 2, no signal was obtained in the absence of acidic treatment and optimum results were obtained between 20 and 35 min. Therefore, in subsequent experiments, samples were treated with 2 N HCl for 30 min. In the next step, we varied the volume of hybridoma supernatant containing anti-5-MeCyd antibodies (about 3 μg/ml specific antibodies) between 50 and 400 μl/10^5 cells. In the previous experiments the optimal volume was found to lie between 150 and 200 μl, as shown in Fig. 3. Under these conditions, analysis by flow cytometry of PBC and P3HR1 cells gave the results illustrated in Fig. 4. Again, unstimulated PBC gave a higher signal than PBC stimulated with PHA and the EBV-transformed cells were less labeled than PBC. Here the specificity of the reaction was demonstrated by the absence of signal with nonimmune isotype immunoglobulins. Treating P3HR1 cells with 5-azacytidine, an inhibitor of DNA methyltransferase, lowered markedly the fluorescent signal. As can be seen in Fig. 5, the decrease in fluorescence intensity collected was proportional to the length of exposure to the analog and a strong recovery of the signal

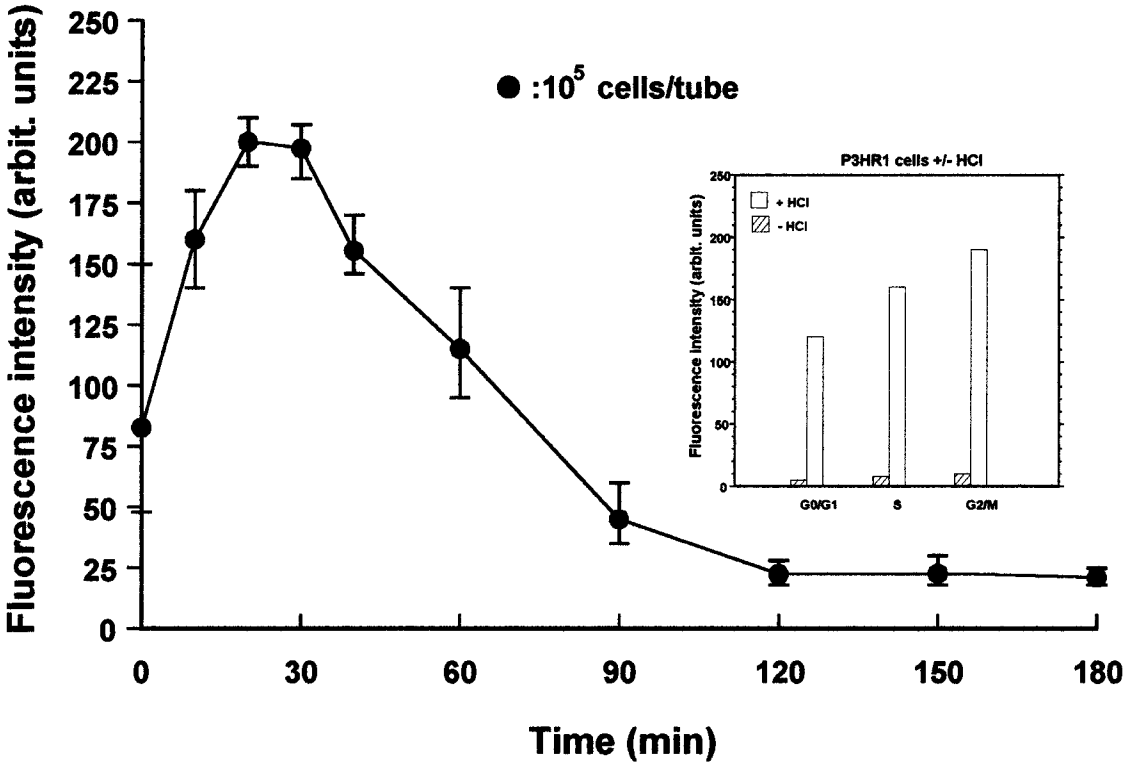

FIG. 2. Treatment of cells with acid. Cells were treated with 2 N HCl for increasing periods of time before immunolabeling and analysis, as described under Materials and Methods. Inset: Phase cycle analysis.

followed the chase. Flow cytometry results are summarized in Table 1.

Differences in fluorescence intensity between samples could originate from variations in the respective degrees of chromatin compaction and from the sizes of nuclei in each sample. Therefore the methylation status of DNA in PBC and P3HR1 cells was assessed by Southern hybridization as described under Materials and Methods. Indexes calculated reached 45 ± 3 for PBC and 76 ± 6 for the EBV-transformed cell line, indicating a high level of hypomethylation: the highest value of hypomethylation in breast tumor cells never exceeded 80 [23]. These data are in good agreement with differences measured between the two cell types by flow cytometry and observed by fluorescence microscopy.

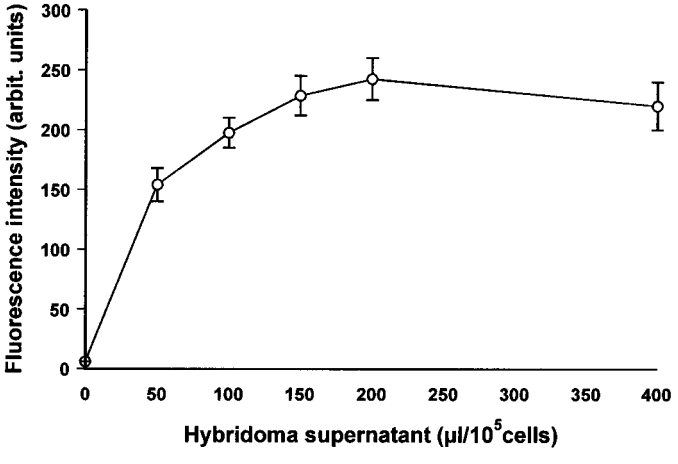

FIG. 3. Optimization of the amount of antibodies to label cells. The volume of hybridoma supernatant volume added to 10^5 cells was varied between 50 and 400 μl. Fluorescence intensities were collected by flow cytometry as described under Materials and Methods.

DISCUSSION

Studying qualitative and quantitative features of the nuclear compartment in normal and transformed cells is a major topic in cell biology as well as in pathology. Classical biochemical methods require extraction of the components to be studied and therefore destroy the nuclear architecture. On the other hand microscopy techniques, either photonic or electronic, allow one to visualize but a limited number of cells. Flow cytometry rapidly became an alternative to the radiolabeling of DNA to determine DNA ploidy and the S-phase fraction of a cell population with no need for cell synchronization. Among the properties of DNA currently studied both in cell biology and in pathology, DNA methylation is an epigenetic alteration which can pro-

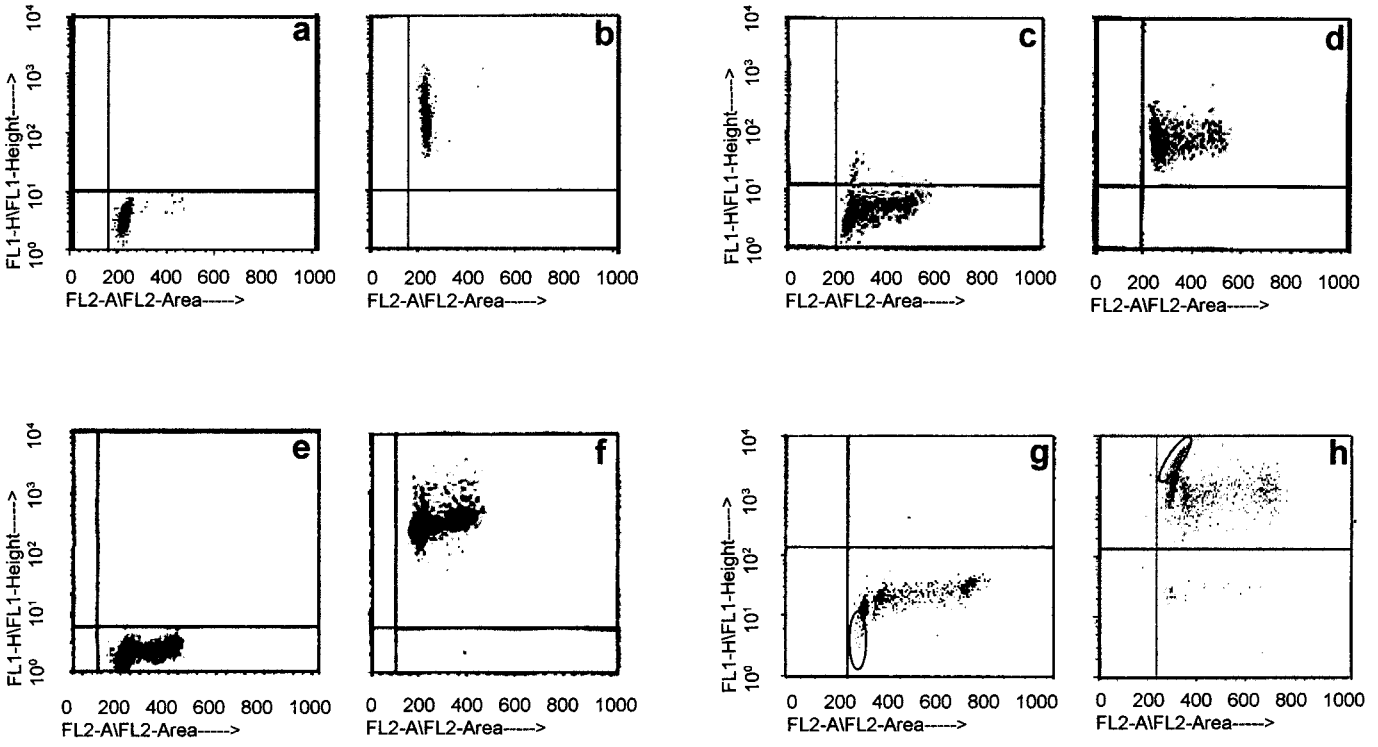

FIG. 4. Examples of dot plots obtained with unstimulated PBC (a and b), with PBC stimulated with PHA (c and d), with P3HR1 cells alone (e and f), and with a mixture of PBC and P3HR1 cells (g and h). (a, c, e, g) No antibodies added; (b, d, f, h) with anti-5-MeCyd antibodies. In g and h the PBC population is circled.

vide valuable information on the establishment and the progression of the malignant phenotype [5, 24]. The possibility of using monoclonal antibodies specifically directed against 5-MeCyd to label methyl-rich regions in metaphase chromosomes and in interphase nuclei prompted us to test their utility in studying DNA methylation in cell suspensions by flow cytometry. For that purpose, one of the major problems was to establish conditions of fixation and permeabilization in which the best compromise between the accessibility of DNA to the antibodies and the preservation of cell morphology could be reached. Among the first results reported in that field were the works describing the immunodetection of 5-methylcytosine, of guanosine [14, 25], and of bromodeoxyuridine in pulse-labeled DNA [26, 27].

Diverse treatments allow the antibodies to reach their target *in situ* [28–37]. We found that permeabilization with saponin or fixation with acetone gave

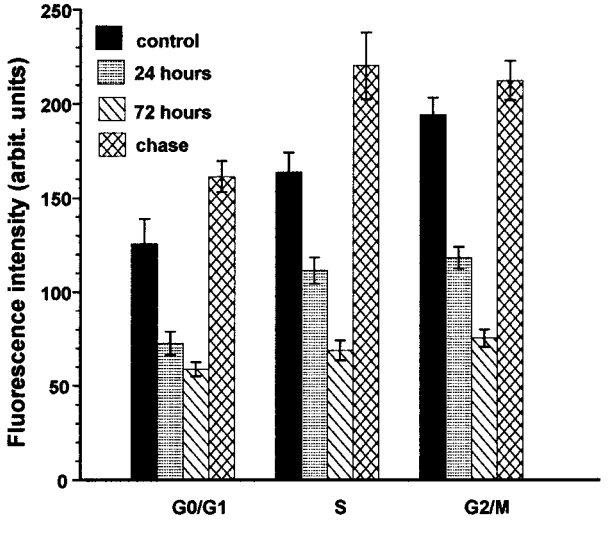

FIG. 5. Effect of 5-azacytidine on the labeling of P3HR1 cells as detected by flow cytometry.

TABLE 1

Flow Cytometry Analysis of PBC and P3HR1 Cells after PI/FITC Labeling

Cells	Treatment	Mean fluorescence intensity (SEM $n = 4$)
PBC	—	424 (81)
PBC	PHA	326 (59)
P3HR1	—	210 (58)
P3HR1	5-azacytidine (24 h)	106 (21)

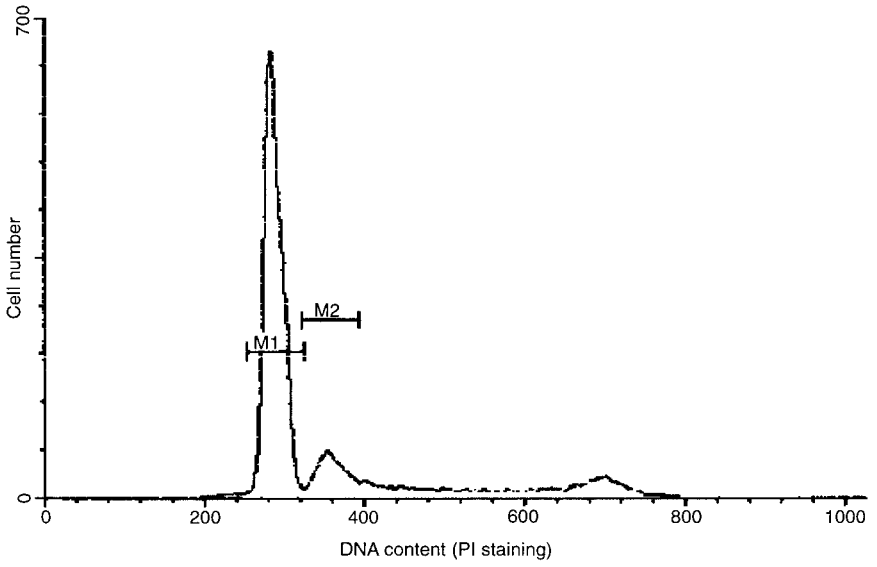

FIG. 6. Ploidy of normal human PBC and P3HR1 cells as measured by flow cytometry after staining with propidium iodide. M1, PBC; M2, P3HR1 cells.

poorly reproducible results, unlike the paraformaldehyde–methanol fixation [38]. In our hands preliminary experiments performed with sodium hydroxide to denature DNA could not be used for flow cytometry. As demonstrated by the pioneering work of Darzynkiewicz [39] with intercalative dyes, treating the nuclei with acid increases the accessibility of DNA. Histones are stripped off the chromatin thereby unmasking the target. Furthermore, depurination of DNA probably induces a lower steric hindrance in the vicinity of 5-MeCyd-rich regions, the methyl groups of which lie in the major groove of DNA. The treatment with acid was optimized to 25 min after testing its effect at regular intervals between 0 and 180 min. Results of this experiment demonstrated that the DNA could not be labeled without treating the cells with HCl and that the length of hydrolysis had to be restricted to 30 min to minimize degradation of DNA.

As seen with fluorescence microscopy and as observed in flow cytometry, cells belonging to a transformed cell line display a lower signal than PBC. Furthermore the transformed cell line population appears to be hyperploid according to the PI profile observed (Fig. 6). Therefore the ratio between FITC and IP signals is lower for the transformed cell line than for PBC. Also the fluorescent signal collected from cells treated with 5-azacytidine was obviously reduced compared to the intensity observed with untreated cells, in accordance with the hypomethylating effect of the analog. When fresh medium was used to wash out the analog, a strong recovery of the signal was observed. This result can be compared to that reported by other authors [40] in which the digestion with *Hpa*II restriction enzyme of DNA extracted from untreated cells and from cells submitted to a pulse–chase treatment gave rise to similar patterns of hybridization with ^{32}P-labeled β-globin fragments. A similar effect was also observed with human tumor cell lines [41] in which, after removal of the drug, the DNA was rapidly remethylated to levels 20% higher than those of untreated cells.

Taken together these results are in accordance with the data published on the global hypomethylation of DNA in tumor cells compared to normal cells [42–46]. However, the respective sizes of the two cell types studied here are different and the difference observed could have been be due to a higher degree of chromatin compaction in the PBC, particularly for cells in the G_0 phase of the cell cycle, thereby inducing a higher intensity of signal than in P3HR1 cells. This is why digestion experiments with restriction enzymes and hybridization with a radiolabeled probe were performed in order to compare results obtained through two different approaches. The similarity of results generated by both techniques demonstrates that the differences in the intensity of fluorescence detected by flow cytometry and observed by fluorescence microscopy between both cell types were not due to differences in the accessibility of chromatin structures to the anti-5-MeCyd antibodies but reveals a real global DNA hypomethylation in the cell line. Provided standards could be established, a semiquantitative estimation of the DNA methylation status might be determined rapidly through this immunochemical approach in samples from unknown origin.

This work was supported by the Fondation de France (grant to A.N.) and the Ligue Nationale contre le Cancer (fellowship to M.H.).

A.d.C. and A.N. are supported by the Galileo French–Italian exchange program. The authors thank Dr. M. Ehrlich for critically reading the manuscript. This work is dedicated to the memory of Dr. J. Huppert.

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Received December 7, 1998
Revised version received February 3, 1999