Environmental Toxicology and Chemistry, Vol. 12, pp. 37–42, 1993
Printed in the USA. Pergamon Press Ltd.

0730-7268/93 $5.00 + .00

SOURCES OF ERROR IN SCREENING BY FLOW CYTOMETRY FOR THE EFFECTS OF ENVIRONMENTAL MUTAGENS

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(*Received* 19 *December* 1991; *Accepted* 13 *March* 1992)

Abstract—Flow cytometry is often used to detect DNA aneuploidy and mosaicism associated with malignancy or genetic damage. Yet DNA aneuploidy and mosaicism detected by flow cytometry may be more apparent than real. In contrast to the DNA mass observed for blood, we consistently found markedly different values and higher variability for DNA mass among other tissues collected from the same animal. Prepared mixtures of blood and other cells generated multiple fluorescence peaks identical to those that might be expected for aneuploid mosaicism. Moreover, analysis of tissues such as feather pulp, which contains a combination of cell types, yielded multiple fluorescence peaks that were not observed when blood alone was analyzed. Thus care should be exercised in classifying DNA values from different tissues as normal or abnormal, because the appearance of supernumerary fluorescence peaks might not always indicate the presence of abnormal cell populations.

Keywords—DNA content False aneuploidy False mosaicism Flow cytometry Environmental screening

INTRODUCTION

A need exists for the development of rapid, accurate, and inexpensive procedures to measure the detrimental effects of environmental contaminants. Among the potential health risks found in the environment are chemical and radioactive materials that can alter the structure of DNA. Structural damage of this type typically has been monitored by cytogenetic procedures. But these are time-consuming, and the number of samples that can be studied is limited. Within the last 20 years, flow cytometry has been used widely to quantify nuclear DNA content [1,2]. Flow cytometry is well suited for the screening of large numbers of samples and is being applied to the detection of DNA damage induced by environmental mutagens and clastogens [3]. DNA aneuploidy has been studied by this method in small mammals after exposure to petrochemicals [4], and in turtles and ducks after exposure to radiation in seepage basins [5–7].

As flow cytometry is used more frequently for screening, it will be necessary to evaluate sources of error within the technology, especially those associated with false aneuploidy or mosaicism in DNA fluorescence distributions. These errors might be due to differential uptake of stain among various cell populations; indeed, we have found that this can occur during routine sample preparation. Thus our objectives in this report were to show that variation can occur in nuclear DNA content among different tissues within individual animals and to illustrate that false DNA aneuploidy and false mosaicism can occur in diverse species. Our data show that supernumerary peaks in fluorescence distributions do not always indicate presence of abnormal (e.g., aneuploid) populations of cells.

MATERIALS AND METHODS

The animals studied and their sources are catalogued in Table 1.

Blood was collected by syringe or capillary tube. In some species of fish, this procedure was accompanied by removing the tail with scissors [8]. Samples were collected into acid citrate dextrose (ACD) solution (Becton-Dickinson, Mountainview, CA, Vacutainer No. 4606). Other tissues were prepared by mincing the tissue with scissors in lysis-staining buffer (see below), followed by filtration through 40-μm mesh.

To eliminate the possibility of error generated

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Table 1. Species surveyed

Scientific name	Common name	N^a	Source	Maintenance	Reference
Ictalurus punctatus	Channel catfish	>350	Stoneville, MS	Spawned and reared in laboratory	[21]
Gambusia affinis	Mosquitofish	>100	Stoneville, MS	Wild-caught, reared in laboratory	
Xiphophorus spp.[b]	Swordtails, platyfish	>100	New York Aquarium	Laboratory-bred for many generations	[8]
Bucorvus abyssinicus	Abyssinian ground hornbill	4	Memphis Zoo	Captive-bred	[22]
Balearica pavonina	Black-crowned crane	7	Memphis Zoo	Wild-caught and captive-bred	[11,22]
Psilopogon pyrolophus	Fire-tufted barbet	2	Memphis Zoo	Wild-caught, reared in captivity	[11,22]

[a]Total number of animals studied by flow cytometry in our laboratory during last four-year period.
[b]Nine species; see reference.

by use of a single instrument, nuclear DNA content of cells was measured with three different flow cytometers – model 753, EPICS V, and PROFILE – all from Coulter Corporation (Hialeah, FL). The procedures are detailed in Tiersch et al. [9]. Before analysis, frozen cells from the channel catfish (*Ictalurus punctatus*) and domestic chicken (*Gallus gallus domesticus*) were thawed and analyzed as a mixture with fresh human leukocytes to calibrate the flow cytometer. For analysis, fresh cells from the animal under study and blood cells from another species used as an internal reference (channel catfish or chicken) were suspended in 0.5 ml of lysis-staining buffer containing 0.1% sodium citrate, 0.1% Triton X-100, 25 μl RNase (1 mg/ml), and 25 μg propidium iodide. The suspension was kept at room temperature and analyzed within 15 min.

Two hundred to 300 propidium iodide–stained nuclei were analyzed per second. The fluorescence of each nucleus was converted to an analog signal, digitized, and used to generate pulse-height histograms from the analysis of at least 30,000 cells. For each fluorescence peak, fractional mode channel and percent coefficient of variation (% C.V.) were calculated by the PARA 1 program (MDADS, Coulter Corporation).

The DNA content per cell was estimated relative to a value of 7.0 pg per leukocyte nucleus for the human male, according to the formula

$$\text{pgDNA} = 7.0 \times (A/B) \times (C/D)$$

where B and C are the fractional mode channels of the reference cells in the A-B and C-D mixtures; A is the fractional mode channel of cells in the sample, and D is the fractional mode channel of leukocytes from a human male.

RESULTS

In each species studied, the nuclear DNA content of blood was substantially different from that of other tissues within individual animals. In the mosquitofish, the surveyed tissues had a mean (±SD) DNA value of 6.4% (±4.4%) greater than the value for the blood of the same animal (Table 2). When the gall bladder was tested, the difference between tissue value and blood value was 18%. Thus sampling of different tissues can yield different values for nuclear DNA content from the same animal. Similar results were observed for the channel catfish (Table 3), although most of the examined tissues were within 3% of the DNA value observed for blood.

Differences in the shape of the fluorescence distributions and in % C.V. were associated with the apparent differences found for DNA content (Tables 2 and 3). Blood samples had narrower peaks with smaller C.V.s than did samples from tissues such as muscle (Fig. 1A, B). When mixed in the laboratory, cells from blood and other tissues from the same animal displayed fluorescence distributions with a pronounced shoulder. In some cases, separate peaks were generated. The shoulders and multiple peaks suggested the presence of aneuploid populations of cells (Fig. 1C).

Fluorescence distributions similar to those in the mixing experiments also were observed in 10 species of fish in which the severed tail was flushed with ACD for blood collection and in pulp samples from the feathers of three species of birds (Fig. 2). The appearance of an additional fluorescence peak or shoulder was variable among the sampled animals, and the relative proportions of cells appearing in either of the peaks in different samples formed a con-

Table 2. Diploid nuclear DNA content of various tissues of the mosquitofish (*Gambusia affinis*) compared to the DNA content of blood cells[a] from the same animal, and percent coefficient of variation (% C.V.) for the fluorescence peaks generated by analysis of the various tissues

		% Difference from blood		
Tissue	*N*	Mean ± SD	Range	% C.V.
Testis	1	−49.9	–	3.96
Mesodermal[b]	7	2.83 ± 3.7	−1.6–6.3	3.54 ± 1.03
Ovary	1	5.4	–	7.32
Embryo	5	5.4 ± 1.9	3.7–8.3	4.13 ± 1.30
Gill	2	7.1 ± 0.7	6.6–7.6	3.78 ± 0.44
Muscle and skin	4	7.3 ± 1.3	6.6–9.3	3.15 ± 0.13
Ectodermal[c]	10	7.7 ± 4.1	3.5–17.7	3.80 ± 1.31
Intestine	1	11.2	–	3.59
Gall bladder	1	18.3	–	3.11
Totals	32	6.41 ± 4.41[d]	−1.6–17.7[d]	3.84 ± 1.18

Measurements were made during the same day with an EPICS V flow cytometer (Coulter Corp., Hialeah, FL) and are reported as mean ± SD; sample size (*N*) refers to number of fish studied for each tissue.
[a]For blood-blood comparisons (multiple samples run in the same day), the differences were negligible.
[b]Includes heart, muscle, and liver. Multiple polyploid peaks with DNA values as large as those corresponding to octaploid and decaploid cell populations were observed for liver.
[c]Includes eye, brain, and skin.
[d]Value for testis not included.

tinuum (Fig. 2C). These mosaic peaks and shoulders were not observed in DNA fluorescence distributions of blood collected with syringe or capillary tube from the same animals.

DISCUSSION

Flow cytometry allows fast, accurate quantification of nuclear DNA content (for identification

Table 3. Diploid nuclear DNA content of various tissues of the channel catfish (*Ictalurus punctatus*) compared to the DNA content of blood cells[a] from the same animal, and percent coefficient of variation (% C.V.) for the fluorescence peaks generated by analysis of the various tissues

		% Difference from blood		
Tissue	*N*	Mean ± SD	Range	% C.V.
Testis	1	−50.3	–	2.65
Tail rinse[b]	2	−0.2 ± 0.0	−0.2	1.98 ± 0.12
Ovary	1	0.8	–	3.10
Anterior kidney	2	1.1 ± 0.6	0.7–1.5	2.38 ± 0.06
Intestine	1	1.3	–	2.45
Gill	2	2.8 ± 0.8	2.2–3.3	3.08 ± 0.02
Ectodermal[c]	8	4.0 ± 6.1	0.5–18.8	2.77 ± 0.43
Mesodermal[d]	6	5.7 ± 5.6	0.6–12.1	2.58 ± 0.19
Totals	23	3.4 ± 4.9[e]	−0.2–18.8[e]	2.63 ± 0.40

Measurements were made during the same day with an EPICS V flow cytometer (Coulter Corp., Hialeah, FL) and are reported as mean ± SD; sample size (*N*) refers to number of fish studied for each tissue.
[a]For blood-blood comparisons (multiple samples run in the same day), the differences were negligible.
[b]The tail was removed and the severed tissues were rinsed with ACD solution to collect cells.
[c]Includes eye, brain, barbel, and skin.
[d]Includes muscle, heart, liver, and spleen.
[e]Value for testis not included.

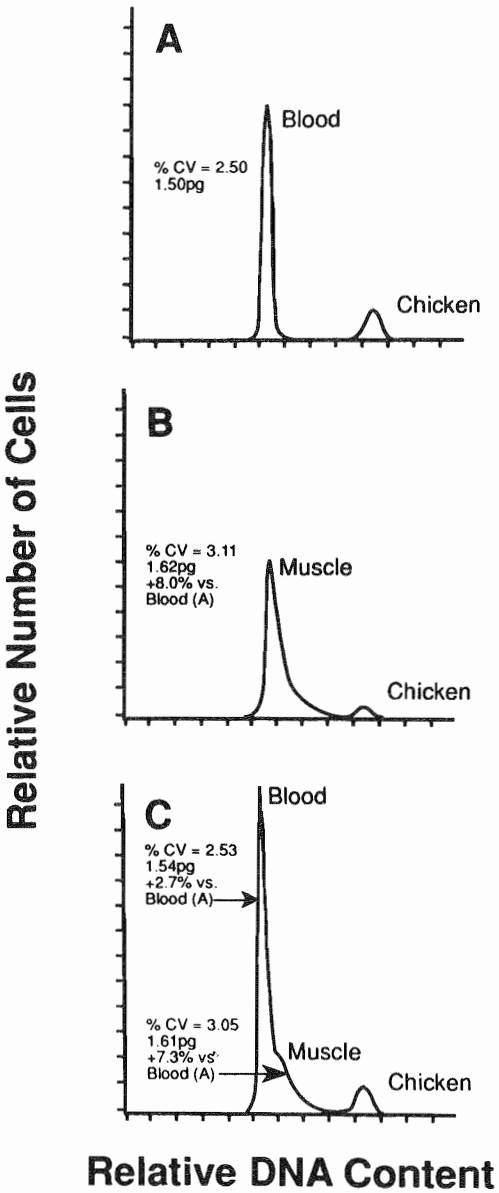

Fig. 1. (**A**) Analysis of nuclear DNA content in mosquitofish blood cells and cells used as an internal reference (chicken). (**B**) Analysis of muscle cells from the same animal that provided blood cells (shown in A); note the larger DNA value of the muscle cells in relation to the blood cells. (**C**) Artificial mixture of blood and muscle cells from the same animal; note the generation of a shoulder on the right side of the fluorescence peak and the higher DNA value of the blood mixture (2.7% increase) compared to blood alone (see A). For each histogram, the vertical axis is relative number of cells, the horizontal axis is relative fluorescence (nuclear DNA content), % CV is percent coefficient of variation, and pg is picograms DNA. Analyses performed with model 753 flow cytometer (Coulter Corp.).

of sex in mammals and birds, for example [10,11]) and screening for genetic damage [12,13]. The method has been used to evaluate natural populations exposed to environmental mutagens. In the studies referred to above, increases in % C.V. and the occurrence of aneuploid cell populations were reported in rodents inhabiting a dump site contaminated with petrochemicals [4] and in turtles and ducks exposed to radiation [5–7].

In our flow-cytometric studies of thousands of animals representing some 200 vertebrate species, we frequently encountered apparent DNA aneuploidy and mosaicism. For the present report, we emphasized the catfish and mosquitofish. Due to their habitats and potential for exposure to and bioaccumulation of mutagenic agents, these fish, along with certain species of birds, would be particularly useful indicators of environmental contamination.

Variation in the DNA content of different tissues has been reported in cytophotometric studies of Feulgen-stained nuclei [14,15]. Within the mosquitofish and to a lesser degree within the catfish, cells from different tissues also displayed different levels of staining after treatment with propidium iodide in our study. Why fluorescence should vary among cells of different tissues is unclear. Unless there is an actual difference in the amount of DNA in the various cells, these differences are likely to be due to staining properties of the different cell types [16,17]. In light of the present results and those of studies with Feulgen cytophotometry [18,19], care should be exercised in classifying values as normal or abnormal when comparing DNA values of different tissues.

Factors that can give the impression of DNA aneuploidy in flow cytometry have been described [20]; they include the autolysis before DNA measurements in cancerous tissues. Our data suggest that the appearance of aberrant fluorescence distributions can be related to the relative proportions of cells from various tissues. When severing the tail of a fish for blood collection, for instance, variable quantities of cells and nuclei are released from tissues such as skin and muscle and mix with cells from the blood. The use of a syringe or capillary tube for blood collection could reduce the potential for producing an admixture of different cell types, compared to sampling techniques that employ rinsing of severed tissues. However, when a small quantity of blood cells is collected, even with a syringe, fluids and cells from other tissues could provide a relatively greater proportion of the total volume than that in the samples composed mostly of blood (our unpublished observations).

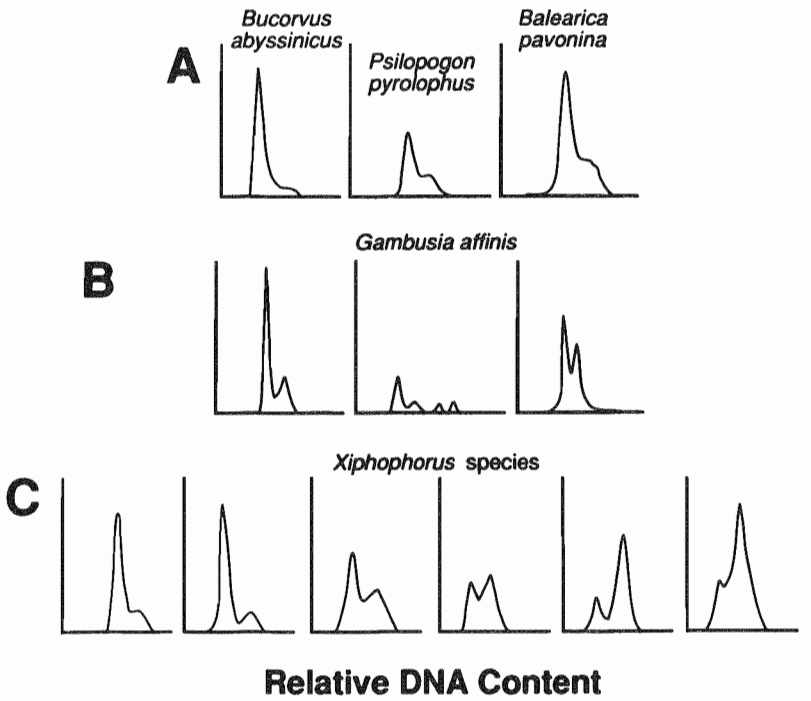

Fig. 2. Examples of data encountered in the analysis of nuclear DNA content by flow cytometry. (**A**) Fluorescence histograms of cells from feather pulp of three bird species showing apparent hyperdiploid (mosaic) cell populations on the right side of the fluorescence peaks. Analyses performed with PROFILE flow cytometer (Coulter Corp., Hialeah, FL). (**B**) Single and multiple hyperdiploid fluorescence peaks found in mixtures of cells obtained by severing the tail in mosquitofish. Analyses performed with EPICS V flow cytometer (Coulter Corp.). (**C**) Variation in the relative proportions of fluorescence peaks of cells collected by severing the tail in fishes of the genus *Xiphophorus*. For each histogram, the vertical axis is relative number of cells and the horizontal axis is relative fluorescence (nuclear DNA content). Analyses performed with EPICS V flow cytometer (Coulter Corp.).

Several different flow cytometers are available at the Flow Cytometry Laboratories of the Molecular Resource Center at the University of Tennessee, Memphis. These instruments allowed direct comparison, which is not routinely available at other institutions. Our observations concerning DNA aneuploidy were consistent regardless of the instrument, which underscores the generality of the phenomenon and the potential for obtaining spurious data.

Acknowledgement—This work was supported in part by National Institutes of Health Grant AI 23479 and a Van Vleet Memorial Fellowship. We thank M. Douglass, K. Kallman, S. Clarki, and D. Queen for help in obtaining samples. Some of the analyses were performed at the Molecular Resource Center, Flow Cytometry Laboratories, of the University of Tennessee, Memphis. We are grateful to R. Chandler for discussion and help with the flow cytometry and to D. Kennedy for his assistance in preparing Figure 1. We thank G. Gee, C. Goudie, and L. Johnson for critical reading of the manuscript.

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