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The use of immunomagnetic separation of erythrocytes in the *in vivo* flow cytometer-based micronucleus assay

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ABSTRACT

The use of sensitive test systems makes it possible to detect weakly genotoxic chemicals and to better define the shape of dose–response relationships, which make it easier to interpret the mechanism behind possible effects.

In this study we have refined the flow cytometer-based micronucleus assay by use of a cytometer equipped with two lasers. Since micronucleated young polychromatic erythrocytes, MNPCE, are very few in number among the cells in peripheral blood, about one or two out of 100,000 erythrocytes, there is always a risk that other cells, doublets or crystals, by mistake will be classified as a MNPCE. With immunomagnetic separation of the very youngest erythrocytes – which are transferrin-positive (Trf + Ret) – prior to analysis, we have obtained an almost pure (>98%) Trf + Ret-population. To clarify whether this separation of cells prior to analysis increases the sensitivity of the already sensitive and further refined flow cytometer-based micronucleus assay, we studied the dose–response towards benzo(*a*)pyrene, B[*a*]P in the low-dose region, 0–30 mg/kg bw. Thirty FVB mice were intraperitoneally injected with B[*a*]P. From the same blood samples collected from these mice, cells were prepared in the two different ways and analyzed in the flow-cytometer equipped with two lasers. The lowest dose of B[*a*]P that can be reliably determined without being overwhelmed by the estimated error was about the same for the two methods, about 7 mg/kg bw, *i.e.* the immunomagnetic separation did not increase the sensitivity. A second study with BalbC mice strengthen**s** the result obtained with the FVB mice. Prior to the low-dose study the optimal sampling time for the two methods was determined. In this case, the water-soloube chemical acrylamide was used. The time courses obtained show almost the same shape of the curves, with a maximum of fMNPCE and fMNTrf + Ret at about 40–50 h after exposure.

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1. Introduction

There are several reasons why it is important to use sensitive test systems in genotoxicity studies, not least to give the possibility to detect weakly genotoxic chemicals. More solid data also facilitate the interpretation of mechanisms behind an obtained effect. Although clear results are lacking, the general opinion is that the dose–effect relationship of genotoxic compounds is linear, *i.e.* each new molecule that we are exposed to also increases the risk that the molecule will bind to DNA, cause a lesion, and end up with a point mutation or a chromosome mutation (single- or double-strand break). As the biomarkers for exposure, *e.g.*, haemoglobin and DNA adducts, do not always reflect the genotoxic effect, it is necessary to obtain information on genotoxicity that is as clear as possible (for a review see Swenberg et al.) [1]. Since there are factors, *e.g.*, DNA repair, that may have an impact on the linearity of the genotoxic response, some *in vivo* studies have been undertaken in order to clarify the shape of the dose–response curve [2–8]. Concerning *in vivo* genotoxicity studies in mammals, it is not easy to clearly demonstrate the shape of the dose–response curve, especially in the low-dose region. There are factors that impact on the statistical power, *e.g.*, different effects between animals belonging to the same dose group, the number of analyzed cells as well as the proportion of false–positive events, classified as positive.

One of the most common short-term assays for the study of *in vivo* genotoxicity is the micronucleus (MN) test. The test with mouse bone-marrow erythrocytes [9,10] is the most developed and most widely used among the MN tests. Since the beginning of the seventies, the method has been further developed. The first attempt to automate the analysis of micronucleated erythrocytes was made by Hutter and Stöhr, who used flow-cytometry [11]. The analysis was made on a flow cytometer equipped with only one laser, allowing the discrimination between micronucleated and non-micronucleated erythrocytes. With the flow cytometer it was, however, not possible to separate older (NCE, normochromatic) and younger (PCE) erythrocytes. By use of a flow cytometer

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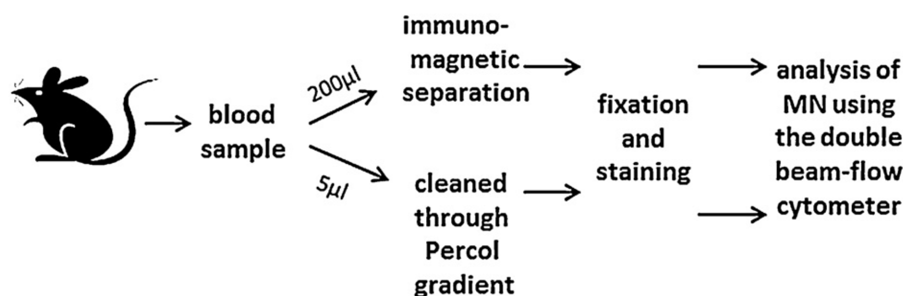

Fig. 1. An overview of the two preparations. From the same sample collected from mice, blood is processed in two different ways prior to the flow-cytometric analysis, *i.e.* the immunomagnetic separation and the Percoll-cleaned-technique.

equipped with two lasers, operating at both multiline UV and 488 nm, Grawé et al. [12] demonstrated that it was possible to discriminate between PCE and NCE, which in turn increased the sensitivity. A few years later, in 1996, Dertinger et al. [13] showed that it was possible to use a flow cytometer with only one laser, and at the same time distinguish very young, transferrin-positive, erythrocytes (Trf + Ret) from other erythrocytes. The solution was to use antibodies connected to fluorescent dyes specific for the Trf + Ret. Since then several studies with mice have been published that used both flow-cytometer techniques.

Although the analyses with the two flow-cytometric techniques described above are restricted to the youngest erythrocytes, PCE or Trf + Ret, the cells are, at the time of analysis, mixed with all the other blood cells, *i.e.* older erythrocytes and nucleated cells. Since micronucleated young polychromatic erythrocytes, MNPCE, are very few in number, approximately one or two out of 100,000 erythrocytes, there is always a risk that subcellular or other debris by mistake will be classified as micronuleated erythrocytes. However, by simply cleaning the cells through a Percoll gradient prior to the analysis of MNPCE this problem was reduced [12]. The proportion of MNTrf + Ret among all other cells is even lower, roughly less than one in a million. To overcome this condition we have further developed the already established flow cytometer-based micronucleus assay by using a cytometer equipped with two lasers [12]. In this study, we have mixed whole blood cells from the mice with anti-transferrin antibodies, anti-CD71, bound to magnetic beads and thereby captured the cells of interest. All the other cells, not Trf + Ret, are then 'rinsed away' from the captured cell population intended for analysis in the flow cytometer. The technique is almost the same as the one already developed for human erythrocytes, published by Abramsson-Zetterberg et al. [14]. We wanted to clarify whether this newly developed technique further increases the sensitivity of the already available flow cytometer-based micronucleus assay. In this study we compare the two techniques. We exposed mice to different low concentrations of the genotoxic compound benzo(*a*)pyrene (B[*a*]P), collected blood, and from the same blood samples prepared the cells in the two different ways. The cells were analyzed in the flow cytometer equipped with two lasers. Based on the dose–response curves obtained, we discuss with which statistical power one can determine the shape of the curves.

2. Materials and methods

2.1. Experimental design and sampling

In the already established and several times published MN assay [2–4,12], the cells are cleaned through a Percoll gradient prior to the analysis. This Percoll-cleaning technique is now compared with the same flow-cytometer technique in which the youngest erythrocytes – Trf + Ret – are subject to immunomagnetic separation prior to analysis, by use of anti-transferrin antibodies, anti-CD71, connected to magnetic beads (see Fig. 1). The study is divided in two separate experiments, a and b. In both experiments the two techniques are used in parallel, and the results are compared.

Experiment (a) was conducted to clarify if the optimal sampling time is the same for Trf + Ret and PCE. Here we studied the time courses. The mice were given a single i.p. injection of acrylamide (AA) dissolved in PBS, at a dose of 50 mg/kg bw. Blood samples were collected at 0, 20, 30, 35, 45, 50 55, 62, and 75 h after injection. The blood sampled from each mouse was divided into two parts, one intended for the immunomagnetic separation method and the other for the Percoll-cleaning technique (Fig. 1). A total of twenty-six male FVB mice, six weeks old, were included in this experiment. We used three mice per group, except for the sampling time at 20 h after injection, where two mice were used. Based on the result from this first study a second time course, containing only 12 mice, three per group, exposed to benzo(*a*)pyrene, B[*a*]P, was determined. Only the fMNPCE was analyzed in this limited study where the sampling times were, 0, 47, 63, and 73 h after injection.

Experiment (b) was conducted to clarify which of the two techniques, as illustrated in Fig. 1, resulted in a dose–response curve with lowest uncertainty. Thirty male FVB mice, seven weeks old, were divided into 10 groups and treated with different low doses of B[*a*]P (dissolved in corn oil). The dose groups received 0, 3, 6, 9, 12, 15, 18, 21, 24, and 30 mg/kg bw. Blood samples were harvested after 47 h. One mouse in the highest dose group died. In another experiment, male BalbC mice, six weeks old, were given a dose of B[*a*]P between 0 and 32 mg/kg bw. In this case only the immunomagnetic separation technique was used.

2.2. Animals

Male Balb-C and FVB mice, aged 6–8 weeks and weighing approximately 20–25 g, were bought from NOVA SCB-AB, Sollentuna, Sweden. The mice were kept in an animal house at the National Food Agency, Uppsala, Sweden. The mice were allowed free access to solid food and tap water, and were kept under 12 h of light and 12 h darkness. Injections had a distributed volume of 10 µl/gram bw. Temperature and humidity were adjusted to be optimal for the animals. Blood samples of about 200 µl were collected from the orbital plexus in heparinized tubes under anaesthesia with Fluothane. Directly after the blood collection, still under anaesthesia, the mice were killed by cervical dislocation. The experiment has been reviewed and approved by the Uppsala Ethical Committee on Animal Experiments, application C334/9.

2.3. Chemicals

The origin of the different chemicals were: Acrylamide (CAS 79–06-01) from MERCK; Benz(*a*)pyrene from Sigma CAS 50-32-8; Percoll from GE Healthcare Bio-Sciences AB, Uppsala, Sweden. The fluorescent dye Hoechst 33342 from Sigma, Aldrich, Sweden and the dye Thiazole Orange from Molecular Probes, OR, USA. Magnetic beads, CELLection™ Biotin Binder Kit, were from Invitrogen Dynal AS, Oslo, Norway; biotin anti-mouse CD71 antibody was from BioLegend, Aachen, Germany; PBS from Statens Veterinärmedicinska Anstalt, Sweden; Glutaraldehyde from TAAB Laboratories, England UK; and Foetal Bovine Serum from Lonza BioWhittaker, Verviers, Belgium

2.4. Flow-cytometric analysis without prior immunomagnetic separation

The procedure with the Percoll-cleaning technique has been described several times before [15]. Briefly, five microliter of heparinized whole blood were layered on a 65% Percoll gradient and centrifuged for 20 min, at 600 × g. After centrifugation, the supernatant containing platelets and the majority of the nucleated cells was carefully aspirated. The remaining cell pellet was suspended in 40 µl PBS and fixed by adding 1.0 ml of a 2% glutaraldehyde solution. The fixed cells were then stored for a few days at 4 °C. On the day of the flow cytometer-analysis the cells were stained with 1.0 ml staining solution per tube. The staining solution contained the fluorescent dyes Hoechst 33,342 (HO) and Thiazole Orange (TO) and was prepared by adding 500 µl HO-stock (500 µM in distilled water) and 80 µl TO-stock (1 mg/ml methanol) to 100 ml PBS. To further refine the method, the staining procedure was partly modified. To some tubes a little more than 1.0 ml of staining solution was added, in order to arrive at blood concentrations as similar as possible between the tubes. The colours of the cell solution obtained disclose the relative concentration

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