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In vivo micronucleus test with flow cytometry after acute and chronic exposures of rats to chemicals

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Abstract

The use of flow cytometry with rat peripheral blood erythrocytes is expected to increase the sensitivity of the *in vivo* micronucleus test and allows assessment of the genotoxic effects at doses that may be equal or close to those relevant to human exposure. However, there was a limitation to the use of rat peripheral blood erythrocytes since the spleen selectively removes micronucleated erythrocytes from circulation. In the present study, the selective analysis by flow cytometry of young MN-PCEs (micronucleated polychromatic erythrocytes or reticulocytes) by use of anti-CD71 antibodies was intended to compensate for the splenic clearance of micronucleated erythrocytes. The young polychromatic erythrocytes have on their surface a specific marker (CD71 antigen) that decreases in density during the maturation process. To investigate the usefulness of the flow cytometric micronucleus analysis combined with anti-CD71 staining of reticulocytes several compounds were tested in acute or sub-chronic treatment regimens. Furthermore, an evaluation was conducted in comparison with the standard rat bone-marrow micronucleus test with additional compounds. The results of acute studies with intraperitoneal application of ethyl methanesulfonate (EMS) (50, 100 and 200 mg/kg) and mitomycin C (MMC) (0.5, 1 and 2 mg/kg), were comparable to data published in the literature. Sub-chronic experiments were performed with cyclophosphamide (CP) (1, 2, 4 and 8 mg/(kg day)), colchicine (6, 8 mg/(kg day)) and mitomycin C (0.1 mg/(kg day)) and showed dose- and time-dependent accumulation of MN-PCEs. Parallel analysis of micronucleus induction in peripheral blood and bone marrow performed with Novartis compounds up to the highest tested dose (5 mg/kg of compound A, 200 mg/kg of compound B and 1250 mg/kg of compound C) showed concordant results. Furthermore, we performed kinetic studies of micronucleus induction in peripheral blood samples obtained at various times after a single treatment with 10 mg/kg CP and with 6 or 8 mg/kg of colchicine. Such experiments gave important supplementary information about the time course of micronucleus induction. Our data suggest that the peripheral blood flow-cytometry micronucleus test can be used for the assessment of micronucleus induction after acute and chronic exposures of rats to chemicals.

Keywords: Rat peripheral blood; Flow cytometry; Micronucleus test; Polychromatic erythrocytes; Reticulocytes; Genotoxic compounds

1. Introduction

The *in vivo* micronucleus test (MNT) is well established as a standard *in vivo* assay for genotoxicity assessment at the chromosomal level [1,2], the mouse being the most widely used rodent species. Peripheral blood erythrocytes have been accepted as an appropriate target for micronucleus (MN) assessment for both acute and cumulative damage [3,4]. However, there is a problem when using rat peripheral blood erythrocytes, since the rat spleen selectively removes MN erythrocytes from the circulation [5]. In a study by Hayashi et al. [6],

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normal (not-splenectomized rats) and splenetomized rats were compared for the induction of MN-PCEs by mitomycin C or cyclophosphamide. The study showed that the incidence of MN-PCEs in the peripheral blood of splenectomized rats was almost identical to the incidence of MN-PCEs in bone marrow and thus the MN cells are not cleared from the blood of splenectomized rats. Data from the Collaborative Study Group for the Micronucleus test [7] suggest that rat peripheral blood may be appropriate for the enumeration of MN, if scoring is limited to the youngest fraction of polychromatic erythrocytes (types I and II) as classified by Vander et al. [8]. By restricting the analysis to the youngest polychromatic erythrocytes (reticulocytes), immature erythrocytes are scored prior to being captured by the spleen [6]. This technique is based on the specific labelling of the young polychromatic cells with the CD71 antigen on their surface, which is lost during the maturation process.

The use of flow cytometry to analyze rat peripheral blood erythrocytes for the *in vivo* micronucleus assay is expected to increase the sensitivity of the test and to allow assessment of genotoxic effects at doses comparable to those that are relevant to human exposure [9–11]. The major advantage of incorporating the MN analysis into conventional chronic toxicology studies is the reduction of the number of animals used and the amount of compound required. This combination would also allow kinetic aspects and general toxicological observations to be used for the interpretation of genotoxicity data.

In this study, several compounds were tested in two treatment regimens (acute and sub-chronic) for their potential to induce MN in the polychromatic erythrocytes of the rat peripheral blood using flow cytometry. Furthermore, with various classic genotoxic compounds, kinetic studies of MN induction in peripheral blood samples obtained at various time points after a single treatment were performed. Such experiments provided important supplementary information about the time course of MN induction.

2. Material and methods

2.1. Animals

Male Wistar rats (age, 8 weeks) were purchased from Charles River Laboratory (Sulzfeld, Germany). The animals were acclimatized for 1 week before the start of the experiments. Food and water were available *ad libitum*. Twenty-five rats were used for each experiment; five rats for each dose group, positive and negative controls.

2.2. Chemicals and reagents

Mitomycin C (CAS number: 50-07-7), colchicine (CAS number: 64-86-8) and ethyl methanesulfonate (CAS number: 62-50-0) were purchased from Sigma–Aldrich (Switzerland), cyclophosphamide (CP; Endoxan-Lyophilisate) (CAS number: 50-18-0) from Baxter AG (Switzerland). Compounds A, B and C are from Novartis. MicroFlow Rat Micronucleus analysis kits were purchased from Litron (NY, USA). The kits contained: solution A (methanol), solution B (heparin), solution C (bicarbonate-buffered saline), solution D (RNase), solution E (anti-CD71 antibody), solution F (propidium iodide (PI)), malaria biostandard blood samples, positive and negative control samples.

2.3. Acute treatment protocols

For the comparative experiments between the classic bone marrow MN test and the flow-cytometry peripheral blood MN test two treatments separated by a 24-h interval were performed. Blood sampling occurred 24 and 48 h after the second application. For the kinetic experiments, a single treatment was performed followed by multiple blood sample collections from the rat tail vein.

2.4. Sub-chronic treatment protocols

For sub-chronic exposures of the rats, daily treatments were conducted for 5, 11 or 13 days. The treatments were separated by 24-h intervals. Blood collection from the rat tail vein was performed every day (for the 5-day experiments) or every 2 days (for the 11- and 13-day experiments), except on weekends.

2.5. Routes of exposure

CP, colchicine and Novartis compound C were given by oral gavage (p.o.), whereas MMC and EMS treatments were by intraperitoneal (i.p.) injection, Novartis compound A was applied intravenously (i.v.) and compound B subcutaneously (s.c.).

2.6. Blood sample preparation

Peripheral blood samples were obtained for flow cytometric analysis according to the protocol of the Rat MicroFlow Plus Micronucleus Analysis Kit (Litron, Rochester, USA). Approximately 120 μl of blood were collected from the tail vein of each animal into a tube containing 350 μl of heparin. Blood samples were maintained at room temperature for no more than 3 h before fixation. Samples were fixed in ultra-cold methanol (−80 °C). For the fixation 180 μl blood was added to a polypropylene centrifuge tube filled with 2 ml ultra-cold methanol, mixed and stored at −80 °C until analysis. On the day of analysis the fixed blood samples were removed from the −80 °C freezer and tapped gently several times to mix the cells, and 12 ml of an ice-cold bicarbonate-buffered saline solution