Technical aspects of automatic micronucleus analysis in rodent bone marrow assays

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Abstract

The mouse bone marrow micronucleus assay is an in vivo test commonly used in the pharmaceutical industry to evaluate the genotoxic potential of new compounds. The test detects agent-induced chromosomal damage or damage of the mitotic spindle apparatus. In this paper the state-of-the-art in automated rodent micronucleus evaluation using computerized image analysis in combination with high-quality slides obtained by the cellulose column fractionation technique is reviewed. The latter allows the effective removal of nucleated cells from rodent bone marrow. It has been found that automatic micronucleus scoring with the Leitz MIAC image analyzer is substantially faster than labor-intensive manual analysis. Automatic scoring can be performed overnight for up to 16 slides. We have been successfully using automatic micronucleus analysis for the testing of new pharmaceutical drugs for more than 3 years.

Abbreviations: MNE, NCE containing micronuclei; MPE, PCE containing micronuclei; NCE, normochromatic erythrocyte; PCE, polychromatic erythrocyte

Introduction

Since the early 1970s, when the pioneers (Matter and Schmid, 1971; Heddle, 1973; Schmid, 1975) developed the mouse bone marrow micronucleus assay to evaluate the genotoxicity of chemical compounds, important improvements of the standard test have been achieved. Attempts to automate the micronucleus scoring were only partially successful because of artifact-producing cell debris originating mainly from nucleated bone marrow cells. The introduction of the cellulose column fractionation technique to remove nucleated cells and debris from bone marrow was a breakthrough in sample preparation (Romagna, 1988). The result is a clean cell fraction containing polychromatic (immature, RNA-positive) and normochromatic (mature, RNA-negative) erythrocytes only. These cell
preparations then can easily be subjected to automatic micronucleus evaluation either by image analysis (Romagna and Staniforth, 1989) or by flow cytometry (Krishna et al., 1993). This paper focuses mainly on the technical aspects of automation by combining the cellulose column fractionation technique with image analysis for automatic evaluation.

Materials and methods

Materials

CD-1 mice were purchased from Charles-River, Wiga, Sulzfeld, Germany.

Microcrystalline cellulose, Sigmacell type 50, α-cellulose fiber (both chemicals from Sigma, St Louis, MO, USA), Hanks’ balanced salt solution (HBSS) without phenol red (Gibco Ltd, Paisley, UK), and FCS mix (fetal calf serum including 25 mmol/L EDTA) were used.

For the micronucleus scoring the Leitz MIAC image analyzer together with a Leitz MEDILUX microscope (Wetzlar, Germany) as an automatic scanning system was used. The Leitz MIAC (Modular Image Analysis Computer) is a computer system using the UNIX-compatible operating system IDRIS with special hardware architecture for real-time image analysis. Morphological transformations on digital images can be performed efficiently by making use of the pipeline architecture of the system, which uses additional video buses besides the standard VME bus. The 68030 CPU board is supplemented by boards for image digitization, video display and image storage and dedicated boards for morphological gray and binary processing of images.

The image capture was achieved using a Hamamatsu CCD camera mounted on the microscope equipped with autofocus, an electronically driven 16-slide stage and automatic lamp regulation. For the micronucleus evaluation, a Leitz 40× PL APO objective with 2× additional magnification was used, together with a 596 ± 16 nm interference filter for the optimum discrimination of the PCEs from the NCEs.

The cellulose column fractionation technique

Column preparation: Equal parts by weight of microcrystalline cellulose, Sigmacell type 50, and α-cellulose fiber were mixed by shaking in a covered beaker for at least 3 min. For each animal (mouse or rat) 300 mg of the cellulose mixture was placed onto the bottom of a disposable plastic syringe (Omnifix 2 ml; B. Braun, Melsungen AG, Germany). Optimal packing was achieved by first tapping the syringe at least 10 times on a hard surface, followed by a slight pressing down of the cellulose mixture with the plunger of the syringe until resistance could be felt in the fingers holding the plunger. At this stage the cellulose mixture should have reached the 1.0 ml mark of the syringe. To avoid parts of cellulose mixture escaping through the outlet of the syringe, an 8- or 10-µm disk filter (Nuclepore, Pleasanton, CA, USA) was placed on a needle, which was then attached to the syringe. The cellulose column is then ready for cell separation.

Fractionation of cells: Femoral marrow cells were collected and suspended using filtered fetal calf serum (FCS) containing 25 mmol/L EDTA (Titriplex III, disodium salt dihydrate; Merck, Germany). It is important to have iso-osmolar conditions and a pH value of 7.4. Before the bone marrow aspirate (about 1 ml for two mouse femora, 1.5 ml for one rat femur) was loaded onto the cellulose column it had to be thoroughly but gently mixed to obtain a uniform suspension of single cells, which was then immediately dropped on to the surface of the column. As soon as the cell suspension had fully soaked into the cellulose mixture, 1.5 ml of HBSS was added to the column surface. An eluate consisting of PCEs and NCEs was obtained after less than 10 min of elution time. The undesired nucleated cell populations remained trapped in the cellulose column.