Journal of Immunological Methods 408 (2014) 137–141

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Technical note

A simple and safe technique for longitudinal bone marrow aspiration in cynomolgus and rhesus macaques

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ARTICLE INFO

Article history:
Received 11 March 2014
Received in revised form 9 May 2014
Accepted 9 May 2014
Available online 17 May 2014

Keywords:
Bone marrow aspirate
Technique
Non-human primate
Macaque
B cell
Plasma cell

ABSTRACT

Longitudinal bone marrow aspirates were obtained aseptically from the humerus of 36 rhesus and 6 cynomolgus macaques by using a 20G spinal needle, introduced through the bone close to the greater tuberosity. All samplings were performed without complications, and the animals showed no signs of pain or infections. The amount of total bone marrow cells obtained from each aspiration varied, in part due to animal-to-animal variation, but the yields were not affected by the sampling frequency or the length of time between each aspiration. The frequency of plasma cells in the bone marrow of each animal was also fairly stable over several longitudinal samplings while a greater, age-dependent, variation was observed between different animals.

1. Introduction

The non-human primate (NHP) model is used for diverse applications in experimental medicine. The NHP species that are most commonly used include rhesus and cynomolgus macaques, which possess high genetic homology to humans (Gibbs et al., 2007). In addition to peripheral blood cells, bone marrow aspirates are readily collected from NHPs as described in studies of mesenchymal and hematopoietic stem cells (Shields et al., 2005; Sharma et al., 2011), investigations of host responses to vaccination (Sundling et al., 2010, 2013), and reports of the recovery of hematopoietic cells for subsequent transplantation (Ueda et al., 2004). The procedures used for propagating bone marrow cells are well described in the literature, but the sampling techniques used to retrieve the bone marrow are often insufficiently described, and in some cases associated with side effects (Llanos et al., 2006). Here, we describe a protocol for collecting longitudinal bone marrow aspirates from the humerus of rhesus and cynomolgus macaques, which in our hands is easy to perform and inflicts minimal harm to the animals. We further describe the yields of bone marrow cells and antibody-secreting plasma cells that can be obtained from longitudinal sampling and we discuss possible sources of variation in cell numbers obtained from this method of sampling.

2. Methods

2.1. Animals and sampling

Bone marrow aspirates were obtained from the humerus of 36 rhesus and 6 cynomolgus macaques derived from three

Abbreviations: NHP, Non-human primate; ASC, Antibody-secreting cell.

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http://dx.doi.org/10.1016/j.jim.2014.05.004
0022-1759/

separate studies, NHP09 (n = 12 rhesus macaques) (Sundling et al., 2010), NHP10 (n = 6 cynomolgus macaques) (Sundling et al., 2013), and NHP11 (n = 24 rhesus macaques) (Martinez et al., unpublished data). Each animal was sampled 2–7 times over the course of 8–37 weeks depending on the design of the study. All macaques were females between three to ten years old. All procedures were approved by the Local Ethical Committee on Animal Experiments in Stockholm, Sweden.

2.2. Bone marrow aspiration

Before sampling, the animals were anaesthetized by intramuscular injection of 10–15 mg/kg of ketamin and 0.5 mg/kg of xylazine. Carprofen was given subcutaneously as analgesia at a dose of 3–4 mg/kg. A strict aseptic technique was used throughout the procedure. Syringes and needles used for the sampling were flushed with a heparin solution. A 20G × 1 1/2″ spinal needle with an attached stylet was inserted through the skin and into the bone 2–3 mm medially to the greater tuberosity of the humerus. By imparting torque, the needle was introduced approximately 15 mm into the humerus until it was firmly engaged in the bone. A 5 ml syringe was attached to the needle, and 1 to 1.5 ml of aspirate was sampled by applying a vacuum in the syringe. With the needle still in place, the syringe was removed and the aspirate emptied into a 9 ml EDTA-coated plastic tube. The procedure was then repeated until a total of 5–6 ml was sampled. Bone marrow aspirates were sampled from alternating arms of the macaques at a maximum interval of 4 weeks (in the NHP10 group; see Section 2.1), giving an 8 weeks period of recovery after sampling.

2.3. Purification of mononuclear cells

The mononuclear cells were purified from the bone marrow aspirates through density gradient centrifugation with Ficoll Paque Plus (GE Healthcare), after which red blood cells were removed by lysis with NH_4Cl followed by extensive washing with PBS. The number of cells was counted and the viability was assessed by labeling with Trypan Blue. Cells were then frozen in fetal bovine serum supplemented with 10% DMSO.

2.4. B cell ELISpot

To detect IgG-producing cells in the bone marrow aspirates, ELISpot plates were coated with 10 μg/ml anti-human Fcγ (Jackson Immunoresearch) overnight at 4 °C. Following washing of the plates in PBS supplemented with 0.05% Tween-20, cells were plated in complete media in a 5-fold dilution series starting at 500,000 cells in the top row and incubated overnight at 37 °C, 5% CO_2. Fresh cells were plated directly, while frozen cells were first thawed in a 37 °C water bath, washed three times in complete media, and left resting over the day before plating. The next day, following washing, the plates were incubated with 0.25 μg/ml biotinylated goat-anti-human Fcγ antibody (Jackson Immunoresearch) for 1.5 h before washing and addition of Streptavidin–Alkaline phosphatase (Mabtech) diluted 1/1000 in PBS and incubation for 45 min. Plates were then washed and spots were visualized by addition of BCIP/NBT substrate (Sigma) for 5 min before stopping the reaction by extensive washing with water. After drying, spots corresponding to antibody-secreting cells (ASCs) were counted using an Immunospot analyzer (Cellular Technology Ltd.) and the amount of ASC per 10^6 plated bone marrow cells was calculated.

2.5. Statistics

All statistical evaluations were done using non-parametric tests. In comparisons including three or more groups or time points, the unpaired Kruskal–Wallis or paired Friedman tests were used followed by Dunn's test for multiple comparisons. When two groups were compared, the Mann–Whitney test was used. Correlations were determined with linear regression and calculation of Spearman *r*. All tests were done using GraphPad Prism version 6 software.

3. Results

3.1. Purified mononuclear cell yields from bone marrow aspirates

A total of 142 bone marrow aspirates were obtained from the humerus of 36 rhesus macaques (100 aspirates) and 6 cynomolgus macaques (42 aspirates). All samplings were performed without complications and the animals showed no signs of pain or infections. The number of mononuclear cells in the aspirates varied between 11×10^6 and 570×10^6 per sample, with a median yield of 55×10^6 (Fig. 1A). No difference in yield was observed between the rhesus and cynomolgus groups. The viability of the cells was generally more than 95%. Memory B cells were previously shown to be undetectable in the bone marrow aspirates, indicating that no contamination with peripheral blood occurs during sampling (Sundling et al., 2010).

3.2. Effect of serial sampling on bone marrow mononuclear cell yields

To determine if serial sampling of the bone marrow would lead to reduced yields of isolated cells, the number of purified mononuclear cells obtained at each sampling was pooled and statistically evaluated using the Kruskal–Wallis test followed by Dunn's test for multiple comparisons (Fig. 1B). No difference was observed between the samplings, indicating that repeated sampling had no detrimental effects on the bone marrow cell yields. To further investigate if the time between each sampling would affect the yield, the macaques were separated into groups based on sampling frequency and interval (Fig. 1C). The 12 rhesus macaques in NHP09 (blue triangles) were sampled 2 times separated by a 4-week interval, the 6 cynomolgus macaques in NHP10 (red circles) were sampled 7 times separated by 4–5 week intervals, and the 18 rhesus macaques in NHP11 (black boxes) were sampled 4 times separated by 4–20 week intervals. Each group was evaluated with the matched-pair Friedman test followed by Dunn's test for multiple comparisons. A significant reduction in the yield of bone marrow cells was observed following the second sampling in the NHP10 group (Fig. 1C), but this likely reflected a temporary drop as the continued sampling time points resulted in higher cell numbers.