Blood Reviews 27 (2013) 91–101

Contents lists available at SciVerse ScienceDirect

Blood Reviews

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

REVIEW

Red cell investigations: Art and artefacts

Giampaolo Minetti [a,b,1,2], Stephane Egée [a,c,1,3], Daniel Mörsdorf [a,d,1,4], Patrick Steffen [a,e,1,5], Asya Makhro [a,f,1,6], Cesare Achilli [a,b,1,2], Annarita Ciana [a,b,1,2], Jue Wang [a,g,1,7], Guillaume Bouyer [a,c,1,3], Ingolf Bernhardt [a,d,1,8], Christian Wagner [a,e,1,9], Serge Thomas [a,c,1,3], Anna Bogdanova [a,f,1,6], Lars Kaestner [a,g,*,1,10]

[a] European Red Cell Society, Europe
[b] Department of Biology and Biotechnology "L. Spallanzani", Laboratories of Biochemistry, University of Pavia, 27100 Pavia, Italy
[c] Station Biologique de Roscoff, UMR7150 Mer & Santé, 29680 Roscoff, France
[d] Biophysics Laboratory, Saarland University, Building A2 4, 66123 Saarbrücken, Germany
[e] Experimental Physics Department, Building E2 6, Saarland University, 66123 Saarbrücken, Germany
[f] Institute of Veterinary Physiology and the Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Zurich, Switzerland
[g] Institute for Molecular Cell Biology and Research Centre for Molecular Imaging and Screening, School of Medicine, Saarland University, Building 61, 66421 Homburg/Saar, Germany

ARTICLE INFO

Keywords:
Erythrocytes
Biochemical analysis
Flux measurements
Flow cytometry
Patch-clamp
Fluorescence imaging
Optical tweezers
Atomic force microscopy

ABSTRACT

Red blood cell research is important for both, the clinical haematology, such as transfusion medicine or anaemia investigations, and the basic research fields like exploring general membrane physiology or rheology.
Investigations of red blood cells include a wide spectrum of methodologies ranging from population measurements with a billion cells evaluated simultaneously to single-cell approaches. All methods have a potential for pitfalls, and the comparison of data achieved by different technical approaches requires a consistent set of standards.
Here, we give an overview of common mistakes using the most popular methodologies in red blood cell research and how to avoid them. Additionally, we propose a number of standards that we believe will allow for data comparison between the different techniques and different labs. We consider biochemical analysis, flux measurements, flow cytometry, patch-clamp measurements and dynamic fluorescence imaging as well as emerging single-cell techniques, such as the use of optical tweezers and atomic force microscopy.

1. Introduction

Contrary to a common belief, the red blood cell (RBC) is a cell type that is neither simple, nor easily obtainable in a pure form. Yet, it is probably the most studied cell type in the history of the life sciences starting with the microscopic observations of Jan Swammerdam in approximately 1660.[1] Nevertheless, as in most other fields of science, contradictory data are common. Sometimes it is possible to unify initially opposing results, e.g., reconciling reports on the electrogenic permeabilities in malaria-infected RBCs[2,3] or on the isolation of lipid rafts from RBCs.[4–6] In other cases, specific issues have not been completely resolved, for example, the number of Gardos channels per RBC[7,8] or contradictory data regarding prostaglandin E_2-induced cation fluxes.[9–11] However, discrepancies often originate from different experimental protocols, inducing different or even opposing degrees of artefacts. Sometimes, artefacts may lead to completely wrong conclusions. This is a serious problem, as revealed in a recent publication[12] in Nature. Here, a standard method intended for the isolation of mononuclear cells (MNCs), based on the density-gradient centrifugation of blood, was mistakenly used to isolate RBCs in an allegedly pure form. This artefact affects the entire paper, but it obviously passed the review process in one of the most prestigious journals.

To avoid this and other common artefacts, as well as to establish a basis for good laboratory practices in RBC research, a subgroup of the European Red Cell Society (ERCS) was formed to initiate standards for a better inter-methodological as well as inter-laboratory comparison

* Corresponding author at: Institute for Molecular Cell Biology and Research Centre for Molecular Imaging and Screening, School of Medicine, Saarland University, Building 61, 66421 Homburg/Saar, Germany. Tel./fax: +49 6841 1626 149/104.
E-mail addresses: minetti@unipv.it (G. Minetti), egee@sb-roscoff.fr (S. Egée), d.moersdorf@mx.uni-saarland.de (D. Mörsdorf), p.steffen@mx.uni-saarland.de (P. Steffen), makhro@vetphys.uzh.ch (A. Makhro), cesare.achilli@unipv.it (C. Achilli), annarita.ciana@unipv.it (A. Ciana), anjwan@uniklinikum-saarland.de (J. Wang), bouyer@sb-roscoff.fr (G. Bouyer), i.bernhardt@mx.uni-saarland.de (I. Bernhardt), c.wagner@mx.uni-saarland.de (C. Wagner), thomas@sb-roscoff.fr (S. Thomas), annab@access.uzh.ch (A. Bogdanova), lars_kaestner@me.com (L. Kaestner).
[1] www.EARCR.nl.
[2] Tel./fax: +39 0382 987 891/240.
[3] Tel./fax: +33 298 2923 82/24.
[4] Tel./fax: +49 681 302 64163/6690.
[5] Tel./fax: +49 681 302 2977/4676.
[6] Tel./fax: +41 44 6358 811/932.
[7] Tel./fax: +49 6841 1626 103/104.
[8] Tel./fax: +49 681 302 6689/6690.
[9] Tel./fax: +49 681 302 3003/4676.
[10] Tel./fax: +49 6841 1626 149/104.

0268-960X/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.blre.2013.02.002

of RBC-derived data. As an initial attempt, here, we present the first "guidelines" for avoiding artefacts in RBC research: In the first part, we discuss the general challenges, such as obtaining pure RBC preparations, experimental conditions in general and the comparison of studies between different species. In the second part, we review a selection of methods in RBC research, discussing possible pitfalls, how to avoid them and the conditions for comparing/combining different methodologies. Obviously, this cannot be a comprehensive selection, but covers a bunch of the most popular methods and emerging technologies.

Our hope is that this report will be useful to all scientists approaching the study of RBCs or considering RBC research, to avoid stumbling into major artefactual conditions and obtaining or concluding the best from the experiments.

2. Ethics

The data presented in this paper has been acquired after approval by the local ethical committees related to the authors institutions.

3. General considerations

3.1. Obtaining pure cell preparations

The vast majority of biochemical studies, but also all other types of cell population measurements, have been carried out, and still are, using bulk suspensions of supposedly pure RBCs. The RBCs are obtained by sedimenting the cells by centrifugation from a sample of whole blood that has been "washed" with variants of a physiologic solution, followed by removal of the supernatant and the thin superficial layer of cells. The latter, the so-called "buffy-coat", is indeed enriched in white blood cells (WBCs), or leukocytes, but these cells belong chiefly to the MNC type, i.e., lymphocytes and monocytes. The most abundant WBCs, however, the polymorphonuclear neutrophil granulocytes (PMNs), tend to remain mixed with the RBCs owing to the similar density of the two cell types, contaminating the RBC sample.[6] The only way of removing most of the WBCs is by filtering the blood with leukodepletion filters. Roughly speaking, if the total content of PMNs per million RBCs is 1000 in whole blood, it will decrease, at best, to 100 in washed blood and to <10 in filtered blood.[6]

3.1.1. Purification of RBCs

A simple and reliable procedure for RBC purification that is suitable for samples of small volumes and easy to implement in every lab is filtration through cellulose, as was originally proposed by Beutler et al.[13] and described in detail in the supplementary material of Achilli et al.[14]

We propose this simple concept as a standard method and good laboratory practice in RBC research. It should be emphasised, however, that filtration might not be applicable in all instances, e.g., for pathological RBCs, because its functioning principle appears to be based largely on the difference in deformability between RBCs and WBCs.[15] The latter are much less deformable than normal RBCs and are therefore retained in the filter for a longer time than RBCs. However, in certain RBC pathologies, RBC deformability is abnormally reduced, and this may result in reduced filterability (hereditary spherocytosis, hereditary elliptocytosis, ovalocytosis, sickle cell anaemia).

3.1.2. Quantifying RBC purity

The task of quantifying low WBC levels is by no means a simple one, and special techniques have been devised for this purpose. As a general remark, microscope counting using conventional haemocytometer chambers is impractical and not sensitive enough. The flow cytometry (FCM) approach is meaningful only if the number of total events counted in each analysis is sufficiently high to reveal 1 WBC per 10^6 RBCs, which implies long analysis times.[16] An extremely sensitive and inexpensive method for the quantification of PMNs in blood samples that can be easily implemented in all labs is the technique of gelatin zymography, as recently adapted.[14]

3.1.3. Consequences of contaminated RBC suspensions

The consequences of having a PMN-contaminated RBC suspension can be deleterious. Two main types of artefacts can result from such a situation: (i) attribution to the RBCs of a component/function that in fact belongs to the PMNs; (ii) damage to RBCs resulting from hydrolases and oxidases released by activated or broken PMNs.

The first issue has already been exemplified in the Introduction. The wrong method used in a recent Nature article[12] for the purification of RBCs results, instead, in the isolation of a fraction of RBCs together with all the PMNs that were originally present in the blood sample, without even reducing the number of PMNs, as would occur if a conventional centrifugation-based wash of the blood and removal of the "buffy-coat" were performed. Fig. 1A indicates the amount of PMNs left by different separation methods.

The artefactual results that originate from PMN hydrolases damaging RBC components are exemplified by the controversy on the isolation and characterisation of lipid rafts from RBCs.[6] The most powerful and constitutively active hydrolases in the PMNs are the serine proteases elastase and cathepsin G. These hydrolases are normally confined at high concentrations in cytoplasmic vesicles (granules) and only released upon cell activation. Detergents can easily free the proteases from the granules. It was shown that even the presence of one PMN per million RBCs is able to release enough proteolytic power to damage, if not fully inhibited, highly sensitive RBC proteins such as ankyrin and protein 4.1.[6]

Another common situation that could give rise to artefactual results is the preparation of "ghosts" from RBCs by hypotonic haemolysis.[17] If the RBCs are contaminated by PMNs and the buffers used are not effectively supplemented with anti-proteases, the RBC membrane proteins will almost certainly be damaged (Fig. 1B, C). The workaround to this problem is the filtration of the blood and the use of freshly prepared lysing buffers containing a working concentration of anti-proteases.

3.2. Experimental conditions

Other factors that must be standardised to be able to compare the obtained data between different laboratories are the temperature, shear stress, medium content, especially traces of serum, and the condition of cells used in the experiments. Furthermore, recent studies emphasise the importance of co-factors and substrates of several receptors, which may contribute to the experimental outcome.

3.2.1. Possible artefacts and their causes

3.2.1.1. The influence of temperature. Temperature-related artefacts include ion misbalance and the ensuing changes in cell volume and Ca^{2+}-dependent processes. Temperature sensitivity depends on the particular approach, but it can be severe, differing, e.g., between different types of ion transporters. The decrease in the activity of ion transporters with a decrease in temperature by 10° (Q10) is approximately 30-fold for the Ca^{2+} pump,[18] approximately 3-fold for the Na^+/K^+ pump[19] and approximately 1.5–3-fold for most of the ion transporter systems.[20,21] Thus, temperature changes may have a pronounced effect on the intracellular Ca^{2+} levels and the Na^+/K^+ distribution. The temperature may not necessarily be fixed at 37 °C in particular experimental settings (e.g., controlling the temperature can be complicate for patch-clamp investigations). However, temperature as a factor has to be taken into account, and the potential side effects must be controlled.

3.2.1.2. Impact of medium supplements. Serum and the multiple biologically active factors it contains, including albumin and factors bound to it, such as interleukins, prostaglandins, insulin and amino acids,