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Light Microscope Observation of Circulating Human Lymphocytes Cultured in Vitro

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ABSTRACT

The purpose of this work was to study the isolation and a light microscopy technique for cultured lymphocytes. Blood samples were obtained by venipuncture with an anticoagulant added and centrifuged in a Percoll density gradient to separate the leukocytes. Lymphocytes were placed in 25 cm^3 tissue culture flasks at 37°C. After culturing, they were fixed and stained with the methods used for blood smears. Results showed that not all fixing solutions and stains were an equally good choice for cultured lymphocytes.

Key words: blood- *in vitro*- lymphocytes- microscopy- morphology- Percoll

INTRODUCTION

Lymphocytes are an important component of the immune system for humans and various animals. They are the only cells that can recognize and distinguish between antigenic determinants, which give them characteristics of an acquired immune response, that is, the specificity of a memory (Abbas et al., 2003). These cells have largely been used in studies of apoptosis due to the striking importance of their apoptotic death during immune system maturation and some diseases (Rathmell and Thomson, 2002). Based on the morphological characteristics, Kerr et al. (1972) developed the unifying concept of cell death as an instrument for the disposal of unwanted cells during embryonic development, cell turnover of proliferating tissues and in pathological situations. There are few preparation techniques for the isolation and observation of lymphocytes. The purpose of this work was to report the details of preparation techniques specific for lymphocytes obtained by venipuncture and maintained in culture.

MATERIAL AND METHODS

Lymphocytes were obtained from samples of peripheral blood, by venipuncture with an anticoagulant (EDTA) (project approved by Ethics in Research Committee of the Medical Sciences College / UNICAMP). Twenty milliliters of each blood sample were centrifuged in a conical centrifuge tube for 15 minutes (1300g) to separate the erythrocytes. The interface between the plasma and erythrocytes was carefully transferred with a pipette into another centrifuge tube with as few erythrocytes as possible, and centrifuged in a Percoll density gradient (Amersham Pharmacia Biotech) for 30 minutes (660g), to separate blood cell types (modified from Hjorth et al., 1981). The highest concentrations of lymphocytes were

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obtained when 100% Percoll was diluted (1:1) in physiological saline solution, thus obtaining 50% Percoll (density (g/mL) -1.06-1.08). The layer containing mononuclear cells (Percoll-50%-density: 1.06-1.08 g/mL) was washed twice in Hanks Solution to remove the Percoll. These cells were ressuspended in RPMI 1640 medium containing antibiotics (streptomycin 10 mg/L and penicillin 1000U/L) and 10% fetal bovine serum (FBS) (Nutricell, Campinas-SP) and incubated at 37ºC for 2 h, for adhesion of the monocytes. The cells in the supernatant were adjusted to 4.5 x 10^5 lymphocytes/mL and placed in 25 cm^3 tissue culture flasks at 37°C.

After culture, cells were collected by centrifugation and washed once in PBS. Then, they were fixed in methanol, methanol-acetic acid (3:1,v/v) (Moura, 1977), 2% paraformaldehyde in PBS for 30 minutes (4°C) or 0.2% formaldehyde in PBS for four minutes (37°C), (modified from McCarthy, 1990), placed on slides, air dried and stained with Leishman, Wright, Panotic dye and May-Grünwald-Giemsa. A large number of slides were prepared with the different methods (n < 10), and the chosen method was used extensively throughout the study.

RESULTS

Both of the fixing solutions of methanol and acid methanol did not conserve the cytoplasm, as can be seen in Figures 1b and 1c. Figure 1d showed an even poorer preservation when the smear was made in saline physiological solution without previous fixation. The cytoplasm was well conserved using concentrations of 2% and 0.2% paraformaldehyde (Figs 1e and 1f). However, the cells decreased in size with both of these fixing solutions, when compared to blood smears (Fig. 1a). The stains tested showed a good contrast for nucleus and cytoplasm in preparations made with blood smears, however, for cultured lymphocytes they did not have same quality of contrast. To obtain better results, the stains were diluted and tested for shorter staining periods, and the best results were obtained with Leishman, at a concentration of 0.2%, applied for a staining period of four minutes at 37°C, as shown in Figure 1f.

A comparison of fixing and staining procedures has been made in Table 1.

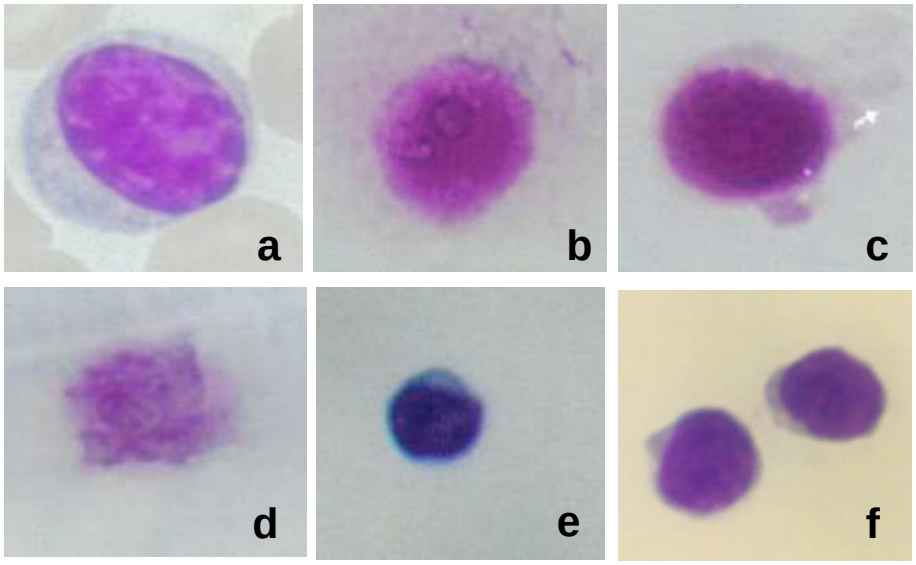

Figure 1 − (a) Blood smear, fixed and stained with Leishman (x1000); (b) Cultured lymphocyte fixed with methanol and stained with Leishman (x1000); (c) Cultured lymphocyte fixed with methanol-acetic acid and stained with Leishman (x1000); (d) Cultured lymphocyte washed in saline solution and stained with Leishman (x1000); (e) Cultured lymphocyte fixed with 2% paraformaldehyde and stained with Leishman (x1000); (f) Cultured lymphocyte fixed with 0.2% paraformaldehyde for four minutes (37° C) and stained with 0.033% Leishman during four minutes (x1000).

Table 1 - Comparison of fixing solutions and stains used for blood cells; + good; - medium; -- poor conservation.

Dyes / **Fixative Techniques**	**Leishman**	**Wright**	**Panotic**	**May-Grünwald-Giemsa**
in vitro				
methanol	--	--	--	--
methanol- acetic acid	--	--	--	--
2% formaldehyde	-	-	-	-
0.2% formaldehyde	+	-	-	-
no fixation	--	--	--	--
blood smear	+	+	+	+

DISCUSSION

In vitro studies are indicated for various types of investigations, including genetic (Geyikoglu and Türkez, 2006) and morphological research (Sell and Costa, 2003). Comparing the materials and methods classically used for blood smears and the preparations made with cultured lymphocytes, it was observed that fixing solutions containing methanol or methanol-acetic acid, which have been recommended for blood smears (Moura, 1977), did not conserve the lymphocytes. These solutions had a fixing quality (denaturing proteins) that was inferior to its capacity to solubilize lipids and destroy the plasma membrane, resulting in cytoplasm loss (Figs. 1b and 1c). To minimize this effect, the slides were prepared using physiological saline solution, in order to extract the culture medium, then air dried and immediately stained, without first applying a fixing solution. The stains used were dissolved in methanol in accordance with the usual methods (Moura, 1977), so that the smears could be prepared using the stain with simultaneous fixative and staining effects, applied for only one minute. As seen in Figure 1d, this method was also not efficient for the cultured lymphocytes, because the stains were not efficient fixing agents, giving inferior results when compared with the other fixing solutions tested (Figs. 1b and 1c). Hence, in relation to fixation, the best results were obtained with paraformaldehyde that showed the best cell conservation (Fig. 1e and 1f). The usual stain for blood smears (May-Grünwald-Giemsa, Wright and Panotic dye) did not result in a good contrast between the cytoplasm and nucleus in the preparations of cultured lymphocytes. As with the fixing solutions, adjustments with lower concentrations and shorter staining periods were made in relation to those recommended for the blood smears. An exception was Leishman stain, which distinguished between the nucleus and cytoplasm at a concentration of 0.033% with a staining period of four minutes (Fig. 1f). However, this only was possible when the fixative concentration of the paraformaldehyde was below 0.2%. The higher concentration (2%) of paraformaldehyde did not give a good contrast, since this fixative induces the basophily of the cytoplasm by making it acidic and staining as strongly as the nucleus, which is basophilic. The paraformaldehyde diminishes the potentially cationic amino acids (which induce the acidophily), whereby they no longer act as ionizing groups or even may be transformed into other compounds (Baker and McCrae, 1966). Paraformaldehyde reduces the number of amino acids that are potentially cationic (indicating acidophily), converting them to non-ionizing groups or other compounds (Baker and McCrae, 1966). Another factor observed for paraformaldehyde was the diminished cell volume when employing either concentration. According to the literature, preparations with this fixative shrank approximately 33%, which may be due to respiration inhibition, changes in plasma membrane permeability or in transport of ions across the membranes (Baker and McCrae, 1966; Penttila et al., 1975; Hopwood, 1985). McCarthy *et al* (1990) reported that the fixed cells could return to their original volume when washed to remove all the fixative, but this was not observed in this research.

In conclusion, our results revealed a marked

difficulty in the preparation methods for the morphological investigations of lymphocytes maintained in culture, which is does not occur in fresh blood samples. The best results were obtained with 0.2% paraformaldehyde, followed by the Leishman stain at 0.033% with a staining period of four minutes at 37° C.

RESUMO

Os linfócitos são células importantes do sistema imune e têm sido largamente utilizados em estudos morfológicos. Entretanto, a literatura sobre técnicas de preparação dessas células é escassa e antiga, especialmente para linfócitos cultivados *in vitro*. Portanto, o objetivo desse estudo foi relatar com detalhes as técnicas de isolamento e microscopia de luz de linfócitos mantidos em cultura. Amostras de sangue foram obtidas por punção venosa e centrifugadas em gradiente de densidade de Percoll, para separar os leucócitos. Os linfócitos foram mantidos em frascos de cultura de 25 cm^3 a 37°C. Após a cultura, as células foram fixadas e coradas de acordo com a metodologia utilizada para esfregaços sanguíneos. Nossos resultados mostraram que nem todos os fixadores e corantes utilizados para esfregaços sanguíneos são uma boa escolha para linfócitos cultivados *in vitro.*

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