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Optimum Ficoll/Hypaque Gradient for Efficient Isolation of Peripheral Blood Monocytes from Camels

[1]Layla Mohamed and [2]M.M. Mukhtar
[1]Department of Bacterial Vaccines Production, Central Veterinary Research Laboratories,
P.O. box 8067 Khartoum, Sudan
[2]Department of Molecular Biology, Institute of Endemic Diseases,
P.O. Box 11463 University of Khartoum, Sudan

Abstract: The viability, Purity and count of monocytes isolated from peripheral blood of camels were assessed after the isolation of these cells on 9, 10 and 12% concentrations of Ficoll/Hypaque. The results obtained showed that 97% viable cells were obtained using the three concentrations of the Ficoll/Hypaque. For purity, 12% Ficoll concentration separated cells were contaminated with others blood cells, while 9 and 10% separated cells were pure. When cells count was applied for the pure isolated cells, the cells isolated on 10% Ficoll/Hypaque concentration showed the best highest count compared to 9% with p = 0.003. Cells isolated using 10% Ficoll/Hypaque well proliferated when stimulated with Phytohaemagglutnin (PHA) mitogen.

Key words: Ficoll/Hypaque, viable cells, peripheral blood, PHA, monocytes

INTRODUCTION

The study of the cellular immune response of the host to various pathogens requires an efficient method for separation of monocytes. Inadequate methods for separation where monocytes could be contaminated with other leukocytes and platelets is not recommended, since secretary products from platelets and other cells can serve to alter the functional tests of monocytes, (Roklin, 1976; Polmar, 1984). Different methods were employed for isolation or enrichment of monocytes populations from blood of different animals (Gorezynski *et al*., 1970; Hayry *et al*., 1973; Julius *et al*., 1973).

Widely used techniques for purification of mononuclear cells from other blood cells employed solutions or substances, which have high density and low viscosity. A commonly used method is that of Ficoll-Hypaque gradient. It is a density gradient separation method, which relies upon differences in cells density.

T cells proliferation is standard method to evaluate cellular immune responses against different pathogens, (Moreno-Lafont *et al*., 2003). Mitogen stimulation of monocytes in vitro is believed to mimic the series of events that occur in vivo following their stimulation by specific antigen. T and B-lymphocytes are activated by different mitogens. Phytohaemagglutnin (PHA) and Concanavalin (Con A) stimulate T lymphocytes. Lipopolysaccharide (LPS) mitogen stimulates B cells. Pokeweed Mitogen (PWM) stimulates T and B cells, (Roitt, 1989).

Nomads who are the mainly camels breeders believed that camels are resistance to many diseases, but scientifically this has never been proved. A technique for isolation of camels monocytes cells is required to study the cellular immune responses in this animal. This study was conducted to develop an efficient method for isolation of pure, viable and adequate number of camel monocytes that could be further subjected to different in vitro immunological studies.

MATERIALS AND METHODS

Ten mL blood samples were obtained from the jugular veins of eight healthy camels in heparinzed vaccutainers.

Separating medium: Ficoll/Hypaque {9% (w/v)} and Ficoll powders were obtained from Sigma. Three different concentrations of Ficoll/Hypaque were used in this study (9, 10 and 12%). For preparation of 10 and 12% Ficoll/Hypaque, different weights of Ficoll powder were added to 9% Ficoll/Hypaque solution. The solutions with added Ficoll powder were then, autoclaved at 121°C for 15 min.

Separation of monocytes: Three mL of each Ficoll concentration was placed in a 15 mL centrifuge tube. Blood obtained from each animal was centrifuged at 1500 rpm for 5 min and the plasma was transferred to a new tube. An equal volume of RPMI -1640 medium (Sigma) was added to sediment cells and well mixed.

Corresponding Author: Layla Mohamed, Department of Bacterial vaccines Production, Central Veterinary Research Laboratories, P.O. Box 8067 Khartoum, Sudan

Seven mL of diluted blood cells were carefully overlaid on the top of each Ficoll concentration. The tubes were centrifuged at 1700 rpm for 25 min at room temperature. The white layers at Ficoll plasma interface were removed, washed in PBS. Viability of the cells was determined by trypan blue (o.2%) exclusion. The viable cells count was done using the method of Leslie and Frank (1991). Briefly the suspension of monocytes cells in trypan blue was placed in a haemocytometer and the central, triple-ruled area of the haemocytometer, was used for the count.

Lymphocytes proliferation assay: To test the response of isolated moncytes (on 10% Ficoll/Hypaque concentration), to mitogenic stimulation, cells were stimulated by PHA mitogen and their proliferative responses were measured using MTT method. For this study, six animals were used. Blood was collected from each animal and monocytes were separated using Ficoll/Hypaque concentration of 10%. For cells proliferation assay, the culture medium used was RPMi-1640 with L- glutamine (Oxoid). This medium was supplemented with 100 μg mL^{-1} streptomycin, 50 μg L^{-1} gentamycin, 100 U mL^{-1} penicillin and 10% heat inactivated fetal calf serum.

A stock solution of MTT [3- (4, 5- dimethylthiazolyl-2)-2,5- diphenyl tetrazolium bromide] (Sigma) was prepared by dissolving 5 mg mL^{-1} in PBS and filtered through a 0.22 μm filter. The solution was stored at 4°C till used. Isopropanol-H CL acid solution was prepared by mixing Isopropanol and 1 mol L^{-1} H CL in ratio of 24: 1. Phytoheamagglutinin (PHA) mitogen (Sigma) was prepared by dissolving 500 μg L^{-1} in RPMI -1640 medium and stored at-20°C.

The monocytes proliferation response was determined by the colorimetric MTT assay described by Mosmann, (1983), with minor modification. Briefly 100 μL of monocytes cells containing 7.0×10^6 cells mL^{-1} from each animal was added to each well of flat bottom 96-well microtetire plate (Corning). Three wells containing cells were stimulated with 40 μL of PHA mitogen, while three more wells were kept as controls without antigenic stimulant. The reagent control was wells containing medium without cells. All wells were adjusted to a volume of 200 μL and all assays were performed in triplicate manner.

The plates were incubated at 37°C under 5% CO_2 atmosphere for 48 h. The plates were then removed from the incubator and 15 μL of MTT solution was added to each wells. The plates were returned to the incubator for overnight period of time. A volume of 150 μL of Isopropanol H CL mixture was added to each well and mixed well.

Table 1: The proliferative response of camel's lymphocytes using PHA mitogen

Camel No.	OD of non-stimulated lymphocytes[a]	OD of PHA stimulated lymphocytes[a]
1	.328±0.00	.750±0.11
2	.288±0.001	.402±0.06
3	.417±0.04	.670±0.16
4	.336±0.01	.501±0.10
5	.360±0.06	.450±0.18
6	.391±0.00	.408±0.01
Mean	.353±0.0185	.530±0.10

Table 2: Monocytes count using different concentrations of Ficoll/Hypaque

Animal No.	Ficoll concentration 9%	10%
1	2.75×10^6	3.70×10^6
2	3.81×10^6	4.90×10^6
3	1.60×10^6	2.27×10^6
4	1.54×10^6	1.57×10^6
5	3.04×10^6	3.17×10^6
6	1.92×10^6	2.4×10^6
7	1.66×10^6	2.11×10^6
8	1.22×10^6	2.08×10^6
Mean	2.19×10^6	2.78×10^6

Measurement of cells proliferation: The proliferative response of camel's monocytes to PHA mitogen was measured using a scanning multiwell spectrophotometer (ELISA reader) (Labsystems Multiskan Ms) using a filter of 492 nm (Cory *et al*., 1991). The results obtained as optical density values (Table 1).

Statistical analysis: To determine the significance of differences between counts of cells isolated on 9 and 10% Ficoll/Hypaque concentrations (Table 2), paired T test was used.

The significant differences betweens the mean values of the optical density of cells and that of mitogen stimulated cells (Table 1) was determining using paired T test.

RESULTS AND DISCUSSION

Study of cellular immunity requires isolation of immune cells that are viable and can survive culture and stimulation. To date few methods were developed for efficient isolation of peripheral blood monocytes cells form dromedary camels. Although Ficoll/Hypaque is commonly used for isolation of peripheral blood monocytes cells from human and several animal species (Julius *et al*., 1973). Its efficiency in isolation of monocytes from camels was not determined. In this study when the viability of camel monocytes cells separated by different Ficoll/Hypaque concentrations (9, 10 and 12%) was examined under the microscope, 97% viable cells were recorded for the three Ficoll/Hypaque concentrations tested. Under the microscope, the cells,

which were separated on 9 and 10% Ficoll/Hypaque concentrations, appeared purred. The cells separated on 12% Ficoll/Hypaque appeared contaminated with other cells e.g. red blood cells, platelets and granulocytes. Therefore 12% Ficoll/Hypaque separated monocytes cells were excluded from further analysis.

When count was applied to monocytes separated on 9 and 10%, the results obtained showed a significant differences between the number of monocytes cells isolated in 9 and 10% Ficoll/Hypaque concentrations ($p = 0.003$) (Table 2). Increasing the Ficoll/Hypaque concentration to 10% resulted in significant increases in number of isolated monocytes that were viable as determined by trypan blue exclusion test. The isolated cells responded well to PHA mitogen stimulation. The mean value of the O.D.of cells stimulated with PHA was significantly ($p<0.05$) higher than the O.D. of the cells without a stimulant, (Table 1).

It is well known that PHA is a potent polyclonal T cell activator (Roitt *et al*., 1989). The magnitude of the proliferative response induced by PHA mitogen in this study could be attributed to the fact that the isolated cells contained both Band T lymphocytes. This study is one of few reports on the mitogenic effect of PHA on camel monocytes cells.

The method described in this study for the isolation of camel monocytes at 10% Ficoll/Hypaque concentration provides an effective tool for isolation of viable, high count and pure monocytes cells from camel peripheral blood that can be used for studying the functional activities of these cells.

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