Assessment of The Purity and Characteristics of Rat Splenic T Cells Isolated by One-Step Discontinuous Gradient of Percoll

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ABSTRAK

DEPAMEDE, N.S. 2010. Pengujian pemurnian dan karakteristik sel T limpa tikus yang diisolasi menggunakan metode gradien densitas Percoll diskontinyu. *JITV* 15(2): 157-164.

Sel-sel T dari limpa tikus (*Rattus rattus*) umum digunakan untuk penelitian imunologis. Akan tetapi sel-sel T tersebut masih banyak terkontaminasi sel-sel lainnya. Untuk itu dibutuhkan suatu metode pemisahan yang praktis dalam satu tahap pemrosesan. Tujuan penelitian ini adalah untuk menguji apakah hasil isolasi sel-sel T dari sel-sel limfosit limpa tikus sebagai hewan model, menggunakan metode gradien densitas Percoll diskontinyu, dapat memberikan pemisahan sel-sel T dengan aktivitas sesuai dengan yang diharapkan dalam suatu penelitian imunologis. Isolasi dilakukan dengan 5 macam gradien Percoll masing-masing 1,052; 1,063; 1,075; 1,085 dan 1,122 g/ml. Uji tingkat kemurnian dilakukan dengan metode *immunocytochemistry* dan *fluorescence-activated cell sorting* (FACS). Sementara uji aktivitas untuk masing-masing fraksi limfosit, dilakukan dengan metode uji proliferasi sel melalui kultur sel yang dipacu dengan *phytohemaglutinin* (PHA) dan *Concanavalin* A (Con A) sebagai pemacu pembelahan sel-sel T. Hasil analisis FACS menunjukkan bahwa dengan teknik pemisahan gradien Percoll diperoleh 4 macam fraksi limfosit. Fraksi I dan II mengandung sel B dan makrofag yang diperoleh dari lapisan gradien 1,052/1,063 dan 1,063/1,075. Fraksi III (gradien 1,075/1,085) mengandung sel T (43,2%) yang terdiri atas CD4 (34,4%) dan CD8 (18,6%), sel-sel B (36,5%), dan makrofag (10,0%). Fraksi IV merupakan fraksi dengan prosentasi ekspresi reseptor sel T tertinggi (76,2%) yang terdiri atas CD4 (63,6%) dan CD 8 (20,0%), sementara sel-sel B ditemukan 20,0% dan makrofag hanya 0,6%. Hasil uji aktivitas sel T yang diekspresikan dari responnya terhadap PHA dan Con A menunjukkan bahwa fraksi III memberi respon tertinggi, sementara fraksi IV dominan dengan tingkat kemurnian sel T tertinggi, memberi respon rendah. Dapat disimpulkan, penelitian ini menunjukkan bahwa metode gradien Percoll diskontinyu dapat digunakan untuk mengisolasi dan memurnikan sel T dalam satu tahapan proses. Sel T membutuhkan sel-sel imun lainnya seperti sel B dan makrofag untuk merespon mitogen secara optimal.

Kata kunci: Percoll, Limfosit, Sel T, Tikus, Mitogen

ABSTRACT

DEPAMEDE, N.S. 2010. Assessment of the purity and characteristics of rat splenic T cells isolated by one-step discontinuous gradient of percoll. *JITV* 15(2): 157-164.

T cells isolated from spleen lymphocytes of rat (*Rattus rattus*) have been commonly used in an immunological research. However the T cells are still contaminated with other splenic cells. Therefore a simple and reliable technique to isolate the T cells in one step process is needed. The aim of the present study was to assess whether T cells isolated from spleen lymphocytes of rat as a model by means of discontinuous density gradient of Percoll could give pure T cells with their activities as needed in an immunological research. Isolation was carried out in 5 density gradients of Percoll i.e. 1.052, 1.063, 1.075, 1.085, and 1.122 g/ml. The purity of the cell fractions was determined by immunocytochemistry and fluorescence-activated cell sorting (FACS) methods. The activity of each lymphocyte fraction was determined through cell proliferation assays by culturing the cells in the presence of phytohemaglutinin (PHA) and Concanavalin A (Con A) as T cells inducers. The FACS results show that fraction I and II were found to contain mostly B cells and macrophages. The fractions were obtained on the layers of gradient 1.052/1.063 and 1.063/1.075. Fraction III (gradient 1.075/1.085) contain T cells (43.2%) which consisted of CD4 (34.4%) and CD8 (18.6%), B cells (36.5%), and macrophages (10.0%). Fraction IV was strongly expressed T cell receptors (76.2%) which consisted of CD4 (63.6%) and CD 8 (20.0%), while B cells were found 20.0% and macrophages just about 0.6%. The activity of T cells as expressed by their respond to PHA and Con A shown that fraction III gave the most positive response, while fraction IV which contained the most ‘pure’ T cells gave less activity. From the present study it can be concluded that the discontinuous density gradient of Percoll method can be used to isolate and purify T cells in one step process. Furthermore, the present study indicated that T cells to proliferate in response to mitogen require other immune cells such as B cells and macrophages.

Key words: Percoll, Lymphocyte, T Cell, Rat, Mitogen

INTRODUCTION

Isolation of human or some laboratory mammals such as mouse, rabbit or rat T cell subsets is important for most studies of T cell function. Recently two mainstream methods of T cell isolation are immunomagnetic cell sorting (IMACS) and fluorescence-activated cell sorting (FACS) (LANCIONI *et al*., 2009). Other methods that have been used extensively are based on density gradient of Percoll as well as Ficoll-Isopaque (GUTIERREZ *et al*., 1979; PELEGRI *et al*., 1995). Using the density gradient method, several subsets of fractions can be attained in just one step process even though crude lymphocytes was used (PELEGRI *et al*., 1995; DAY *et al*., 2008). In some studies, certain degree of T cell purity is required (OUABED *et al*., 2008). For this purpose the methods of IMACS and FACS are commonly used. However these methods need an isolation process before the cells are treated by IMACS or FACS. Since density gradient of Percoll method is able to purify lymphocyte subset from crude lymphocyte extract in single step, this method is still used continuously up to now. However lack of information has been reported regarding degree of purity of the isolated lymphocyte, especially T cell subsets, as well as their nature in response to some mitogens (LANCIONI *et al.*, 2009). The present study was carried out in order to guarantee whether lymphocyte subsets isolated by discontinuous gradient of Percoll has purity and express activity fit to the experiments set.

MATERIALS AND METHODS

The present study was conducted at Department of Animal Sciences, and Department of Microbiology and Immunology, the University of Adelaide, South Australia, as part of a post graduate research program.

Animals

Inbred Dawley rats (*Rattus rattus*) were used as donors of splenic lymphocytes. The rats were adult males, sexually mature, weighing no less than 300g, and housed under standard conditions with food and water provided ad libitum.

Chemicals

Percoll was purchased from Pharmacia Biotech, Amersham. Mouse anti-rat α/β TcR (R73), CD4 (W3/25), CD8 (Ox8) and B cell (Ox33) monoclonal antibodies (mAb), and the fluorescent isothiocyanate-sheep anti-mouse immunoglobulin antibody (FITC-SHAM) were purchased from Serotec Ltd, Oxford, England. Mouse anti-rat mac-1 (CD11b) (WT. 5) and anti-Giardia (IB5) monoclonal antibodies were generously provided by Dr Graham Mayrhofer, Department of Microbiology and Immunology, The University of Adelaide. For cell culture medium, RPMI 1640 was purchased from Cytosystems. Heat-inactivated foetal bovine serum, glutamine solution (200 mM, 2.92%), and Monomed-A (serum replacement) were produced by CSL. Concanavalin A, *Canavalis ensiformis* (Con A), Phytohemagglutinin (PHA), and trypan blue stain (0.4%) were purchased from the Sigma Chemical Company, St. Louis, MO, USA. Tritiated [^{3}H] thymidine (specific activity 1.04TBq/mM) and Biodegradable Counting Scintillant (BCS) were purchased from Amersham Australia. The BCS scintillant was used in conjunction with a 1215 Rackbeta II (LKB, Wallac, Findland) β-counter.

Negative selection systems for $CD4^+$ and $CD8^+$ rat T lymphocyte separation kits were purchased from StemSep™, StemCell Technologies, Inc., Vancouver, Canada.

Spleen cells preparation

The spleen was aseptically removed from adult male Dawley rats euthanized with CO2 and placed in a petri dish containing RPMI 1640 (10 ml) supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), heat-inactivated foetal bovine serum (10%), and L-glutamine (2 mM). This medium was designated as RPMI 1640 complete medium. The spleen was dissected and the content was flushed into the petri dish by injecting medium into the spleen. The splenic cells were then transferred into a 12 ml tube, and allowed to stand for five minutes. The free cells were then placed in a 50 ml conical tube (aggregated cells were discarded), and the tube was centrifuged at 500 x g for five minutes at 20° C. The supernatant was removed and the cell pellet resuspended firstly in 2 ml of the lysing solution (the mix of 8.26g ammonium chloride, 1.00g sodium bicarbonate and 0.04g EDTA in 100 ml Milli-Q water). The rest of the lysing solution (8 ml) was then added and the tube was left to stand for exactly 5 minutes at room temperature.

RPMI 1640 (20 ml) supplemented with penicillin (100 IU/ml) and streptomycin (100 µg /ml) (designated as RPMI 1640 without serum) was added, and the suspension was then centrifuged at 500 x g, 20°C for five minutes. The cells were washed once in 20 ml RPMI 1640 without serum, after which the cells were resuspended with one ml of RPMI 1640 complete medium. Finally the cells were counted and their viability was examined by assessing cell exclusion of 0.2% trypan blue before undergoing further separation.

Table 1. The actual amount of ingredients used to prepare the density gradient of Percoll for the final working solution of 40 ml

Desired density (p) (g/ml)	Working solution (V) (ml)	NaCl (1.5 m) (ml)	Original percoll (Vo) (ml)	Milli-Q water added (ml)
1.052	40	4	14.22	21.78
1.063	40	4	17.60	18.40
1.075	40	4	21.28	15.72
1.085	40	4	24.36	11.64
1.122	40	4	35.76	0.24

T cells isolation using Percoll gradient

Percoll gradient preparation. The density gradients of the Percoll used were 1.052, 1.063, 1.075, 1.085 and 1.122 g/ml. These density gradients were made up by diluting neat Percoll with 1.5 M NaCl, based on the calculation of the following formula (ANONYMUS, 1985):

$$Vo = \{V \times [p - (0.1 \times p10) - 0.9]\} / (po - 1).$$

Where:
Vo = the volume of the original Percoll (ml)
V = volume of the final working solution (ml)
p = desired density of the final solution (g/ml)
po = density of the original Percoll (1.13 g/ml)
p10 = the density of 1.5 M NaCl (1.058 g/ml).

The volume of 1.5 M NaCl used was 10% of the desired volume of working solution, and sterile Milli-Q water was added to raise the final volume to that of the working solution needed. All of the preparation was undertaken using sterile procedures.

Spleen cells (not exceeding 1 x 10^8 cells/ml) were centrifuged in RPMI 1640 complete medium in a 30 ml polysulfone Oak Ridge centrifuge tube. The supernatant was discarded and the pellet was resuspended in 2 ml of complete medium. A discontinuous gradient was prepared by aspirating the lowest density gradient (1.052 g/ml, 4 ml volume) into the tube via a single lumen polyethylene tube (Figure 1).

Successive fractions of the higher density solutions (4 ml volumes, 8 ml for the 1.063 g/ml density gradient) were then added beneath the top layer, thereby preventing mixing of the gradients. The cell solution was then placed on top of the gradients (Figure 1). The gradient was centrifuged at 2000 x g in a bench-top centrifuge (Beckman GPR centrifuge, Beckman) for 10 minutes at 20°C.

The cells at the interface of each layer were carefully removed and washed twice in RPMI 1640 without serum. Cells remaining in the interface between 1.052 g/ml and 1.063 g/ml Percoll density, were designated as 'fraction I', cells between 1.063 g/ml and 1.075 g/ml Percoll density as 'fraction II', cells collected from the interface between 1.075 g/ml and 1.085 g/ml Percoll density as 'fraction III', and cells collected from the interface between 1.085 g/ml and 1.122 g/ml Percoll density were designated as 'fraction IV'. The cells were then prepared for further analysis and characterisation. Isolated T cells viability was determined by trypan blue exclusion.

Assessment of the purity and specific characteristics of isolated T cells were carried out by immunocytochemistry and stained for surface immunoglobulins with a method of single colour fluorescence activated cell sorting (FACS) labelling (BRIDEAU *et al*., 1980, PELEGRI *et al*., 1995) in order to determine the cellular constituents.

Six mouse-anti rat monoclonal antibodies were used to label the lymphocyte fractions, namely anti-α/β TcR (R73) (HUNIG *et al*., 1989), anti CD4 (W3/25) (WILLIAMS *et al*., 1977), anti-CD8 (Ox8) (BRIDEAU *et al*., 1980), anti- B cell (Ox33) (WOOLLETT *et al*., 1985), anti-Mac-1 (CD11b; WT.5) (SPRINGER *et al*., 1979), and anti-Giardia (IB5) as a negative control. This assessment enabled identification and quantification of the cell types isolated in each fraction.

Each lymphocyte cell suspension (1 x 10^6 cells) was aliquoted (1 ml) into FACS tubes on ice. The cells were centrifuged at 250 x g at 4°C for seven minutes. Supernatants were completely removed using a Pasture pipette connected to a vacuum pump, and then desired monoclonal antibodies (50 µl each) were added to each pellet. The pellets were then resuspended by gently vortexing. The suspensions was were incubated on ice and vortexed every 15 minutes. After 45 minutes, 3 ml of ice-cold PBS containing 1% FCS was added to each tube, the suspension resuspended and centrifuged as above. After removing the supernatants, the cells were washed two times in washing solution (PBS containing 1% FCS and 0.01% sodium azide).

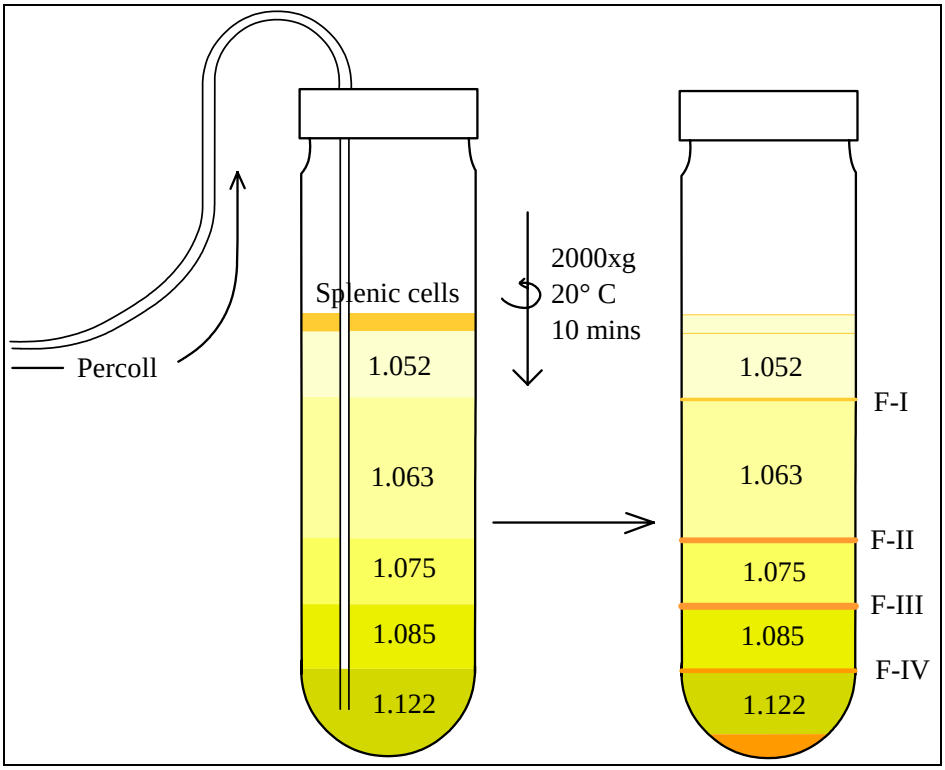

Figure 1. Illustration of separation of T and B lymphocytes from spleen cells on a discontinuous gradient of Percoll in the ranges of density 1.052-1.122 g/ml. F-I, F-II, F-III, and F-IV: Fractions I, II, III, and IV

Following these processes, the conjugate (50 µl, FITC-SHAM in a 1 : 100 dilution with PBS supplemented with 1% normal rabbit serum, NRS) was added, and the cells were resuspended and incubated for one hour on ice. After incubation, the cells were washed twice with two millilitres of washing solution. Following the second wash, the pellets were resuspended in one millilitre of fixative (FACS fixation solution i.e. 1% (v/v) paraformaldehyde) and stored in a dark room at 4°C for 24 h. The cells were then resuspended and analysed by flow cytometry using a FACS Scan (Becton Dickinson, UK).

In order to decide which fraction would give optimum immunomodulatory action, isolated splenic lymphocytes were assayed by means of cell proliferation assay (KOHAMA *et al*., 2003; DEPAMEDE, 2005). The lymphocytes obtained from each Percoll density fraction and from unfractionated spleen cells were cultured in the presence of various concentrations (1.6 to 50 µg/ml) of Concanavalin A (con A) and phytohemagglutination (PHA). Both of these mitogens are known to stimulate T-cell proliferation (TIZARD, 1992; JANEWAY *et al*., 2005). Cell proliferation was assayed through [^{3}H] Thymidine incorporation and the proliferative expression was measured using standard scintillation counting in a 1215 Rackbeta II Beta-Counter machine.

Data analysis

Quantitative data were tabulated and analyzed and Student's *t* test was used for single pairwise comparisons, especially for the response of separated cells against different mitogens. Values of $P > 0.05$ were not considered significant. Qualitative data are visually expressed as presented in FACS results.

RESULTS AND DISCUSSION

Studies on the isolation and characterisation of T cell subsets are important for understanding protective immunity against pathogens mediated by CD8 as well as CD4 T cell activities. This is due to CD8, which function as cytotoxic T lymphocytes, recognise peptides derived from cytoplasmic pathogens, while CD4 recognise microbial antigens that are ingested from the host extracellular (BLANCO *et al*., 2001; JULEFF *et al*., 2009; ABBAS and LICHTMAN, 2009). Therefore succeeded in the isolation of CD8 or CD4 T cells will help researchers to carry out study in more detail on the role of T cell subsets against pathogenic microbes.

In the present study, cell viability of isolated T cells was more than 95% as determined by trypan blue exclusion and very few erythrocytes (less than 5%) were presence after erythrolysis treatment. Cell viability

was even higher following Percoll fractionation (> 98%).

Analysis using FACS method show that fraction I and II were found to contain mostly B cells and macrophages. Fraction III contained mostly T cells (43.2%) which consisted of CD4 (34.4%) and CD8 (18.6%) positive T cells, B cells (36.5%) and macrophages (10.0 %) (Figure 2). The most "pure" T cells were observed in fraction IV which strongly expressed T cell receptors (76.2%) and which consisted of CD4 (63.6%) and CD8 (20.0%) positive cells, with 20.0% B cells, and 0.6% macrophages (Figure 3). The higher proportion of $CD4^+$ T cells compared to $CD8^+$ T cells subset in this study is in complete agreement with the previous study of WESTERMANN *et al*. (1990) who reported that in the blood and other connective tissues $CD4^+$ T cells subset represents the major lymphocyte subset.

Assessment of immunomodulatory of T cells and their subsets were carried out by testing their responsiveness against Concanavalin A (Con A) or phytohemaglutinin (PHA). These substances have been well known as inducers for T cell proliferation. The results show that the combination of macrophages and lymphocytes in fraction III were found to give the optimum response to Con A and PHA while fraction IV which consisted of highly purified T cells and very few macrophages were poorly activated by mitogens (Figure 2ab). Similar results have also been reported by GUTIERREZ *et al*. (1979) and MAYRHOFER *et al*. (1986), who revealed that highly purified T cells respond poorly to T cell mitogens. This phenomenon can be accepted since it has previously been demonstrated that macrophages are needed to optimise mitogen-induced T-cell proliferation. Therefore it is said that this may be the reason that fraction IV, which consisted of 'pure' T cells, gave a poor response to both Con A and PHA mitogens. To induce T cell proliferation, PHA required the presence of accessory cells bearing HLA-class II such as macrophages (KERN *et al*., 1986; JANEWAY *et al*., 2005; LANCIONI *et al*., 2009).

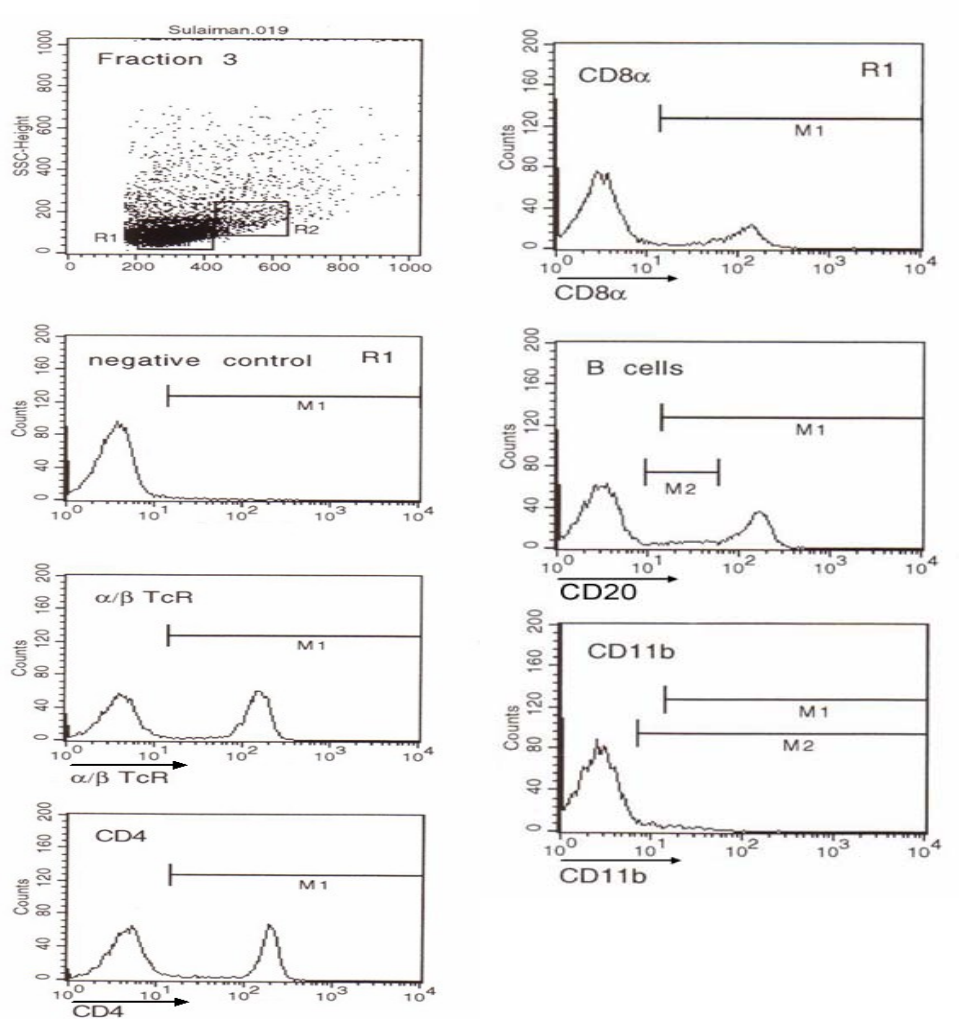

Figure 2.a. Expression of surface markers on cells in Fraction III

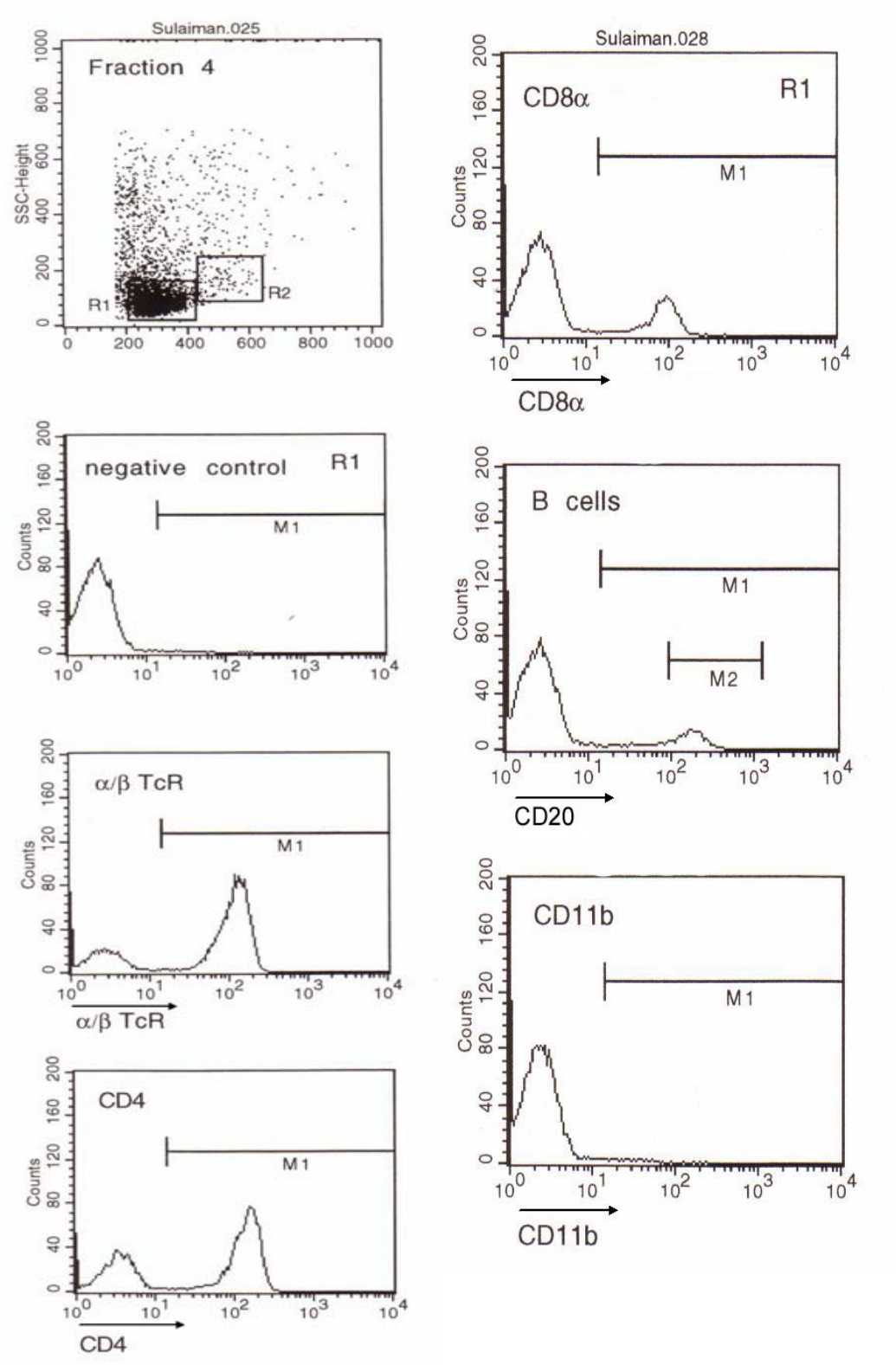

Figure 2.b. Expression of surface markers on cells in Fraction IV

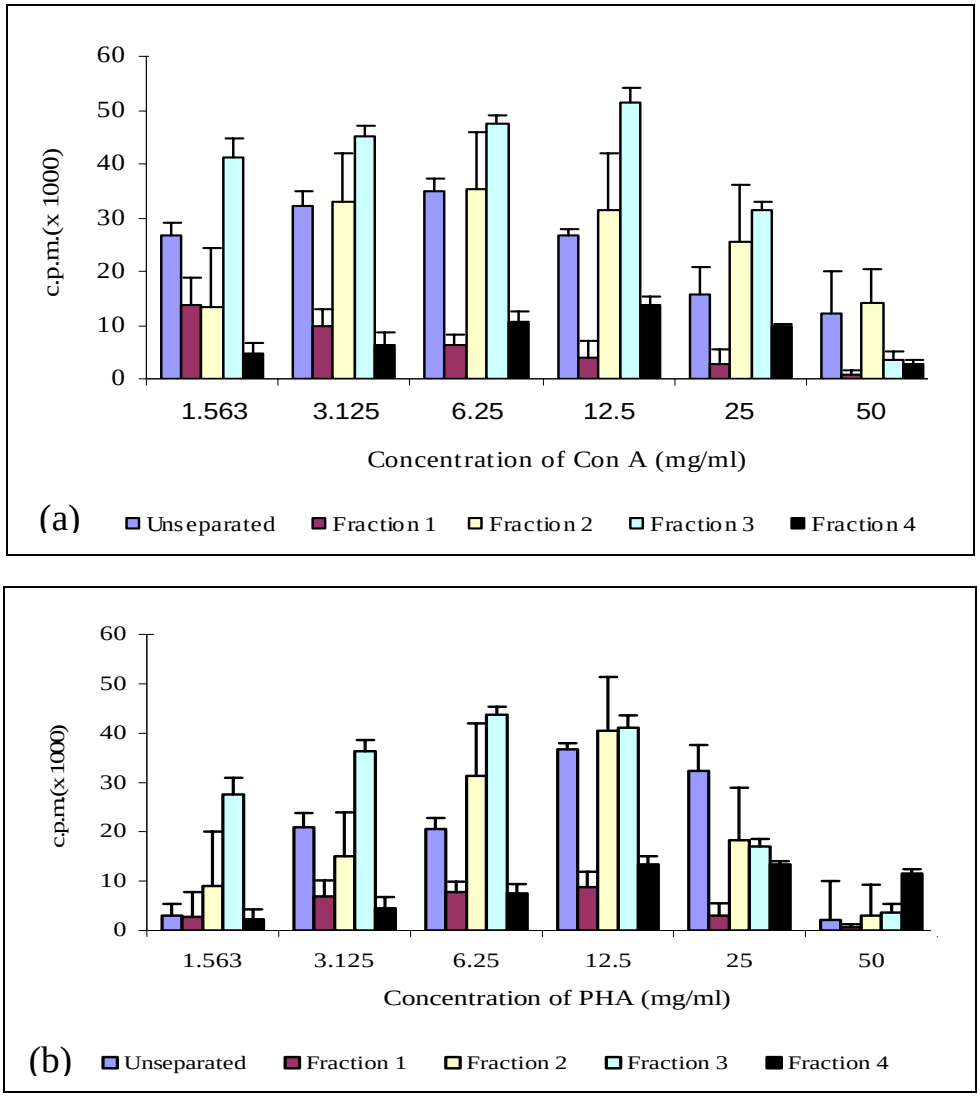

Figure 3. Response of unseparated, and fractionated rat splenic cells separated using a discontinues gradient of Percoll, to several concentrations of (a) Con A or (b) PHA. (Values are Mean ± SEM; n=3; c.p.m. = count per minute)

Recently LANCIONI *et al*. (2009) have also reported that maximum CD4 T cell proliferation was reached when re-addition of low number of accessory cells was added into almost pure CD4 T cells. In addition LANCIONI *et al*. (2009) mentioned that highly purified resting CD4 T cells have stringent co-stimulation requirements.

CONCLUSION

The present study shows that the method of purification by means of gradient density of Percoll could be carried out from crude splenic lymphocytes, which gave several subsets of lymphocyte. The method was able to give highly purified T cells.

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