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The Comparison *in vitro* Study of IgM Production Between Spleen and Peritoneal B-1a Cells

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ABSTRACT

According to the importance of B-1 cells to produce natural antibodies specially against polysaccharide and other bacterial carbohydrate it needs to investigate the possibility of *in vitro* production of IgM by stimulated culture cells with bacterial endotoxin. Isolated lymphocytes from peritoneal lavage, spleen perfusion/disintegration and cardiac puncture from Balb/c mice was purified by density gradient. Cultured cells were stimulated by 50 μg mL^{-1} Lipoplysaccharid (LPS) and collected supernatant from 3 stages of culture times (24, 48 and 72 h) investigated for IgM by ELISA. Immunophenotyping assay has performed by direct Immuno Flourescence (IF) technique and proliferation rate was determined by 3-[4,5-dimethylthiazol-2-Y1]-2,5-diphenyltetrazolium (MTT) technique. IgM concentration in 3 sources of cultured cells were significantly more than control groups ($p<0.001$) specially in 24 h in peritoneal lymphocytes comparison with another times ($p<0.001$). Also cellular vitality was more significant in spleen lymphocyte in 24 h in comparison with peritoneal lymphocyte. Spleen and peritoneal B-1 lymphocyte had more activity and vitality force to produce IgM in 24 h in comparison to control group ($p<0.05$). High level of CD5 expression in B-1 population had observed in peritoneal cells. The obtained results show that spleen and peritoneal B lymphocytes are the main source for IgM antibody against polysaccharide ligands. This potent secretion may be useful as a source of spontaneous and stimulated high level of IgM. It may be as an instrument system for filtration or eluting the media and solvent from LPS contaminations.

Key words: B-1 lymphocytes, spleen, peritoneum, IgM, lipopolysaccharide, MTT, ELISA

INTRODUCTION

B-1-cells differ from conventional B-2 cells in their surface phenotype, anatomical localization, self- replenishing and V_H usage of IgM (Moon *et al*., 2004). B-1 cells are the main provider of natural antibodies against capsular high molecular weight polysaccharides components of extra cellular bacteria in the early stage of defense (Montercion-Rodriguez *et al*., 2006). They secret the most IgM antibody as an important host defense immunoglobulin (Hsueh *et al*., 2002). In the mouse, these population of B cells known as B-1a cells, that contributes in innate immunity in early response to bacterial and viral invasion. These cells also produce natural antibodies mostly IgM independence before exposure to antigens (Hsueh *et al*., 2002). Among the 5 classes of IgM in humans and mice, IgM is unique in its physiology and structural properties. IgM is the first class

of antibodies produced during ontogeny. Also this natural IgM that produce spontaneously, tends to be poly reactive to both endogenous and exogenous conserved bacterial surface component and released endotoxin such as lipopolysaccharide (Boes *et al.*, 1998). In adult mice, B-1 cells produce naturally, the main portion of IgM. They differentiated in lamina properia, pleural and peritoneal cavity and localized there characteristically (Boes *et al.*, 1998). B1 cells differ from conventional B-2 location and self renewing. The progenitors of B-1 cells are abundant in the fetal omentum and liver but are missing in bone marrow of adult animals but enriched in the peritoneal cavities and low frequency in the spleen is seen. They are virtually absent from Lymph nodes, Payers patch and peripheral blood, where most conventional B-2 cell are localized. B-1 cells are categorized into B-1a that express CD5 and B-1b cells that expresses surface markers similar to those of B-1a cells except for the low expression if any of CD5 (Boes *et al.*, 1998). B-1 cells have primarily role to secrete natural IgM antibodies (Boes *et al.*, 1998). They activated by low concentration of LPS than B-2 cells (Tumang *et al.*, 2004). LPS could accumulate B-2 cells preferentially. A threshold level for B-2 cell response (significant increase in cell number) was found to be 100 ng mL^{-1} LPS in contrast to the B-1 B cell subset which were only significantly different to the unstimulated cells which stimulated with 50 μg mL^{-1} LPS. Significant response in both subsets following stimulatory with 1 and 10 μg mL^{-1} LPS were seen (Julia, 2006). These investigations made profitable data which advocate the reflection that B-1 and B-2 cells could respond separately to LPS. It is still possible that B-1 cells more sensitive to low concentration of LPS. The result presented together by other investigators, suggests that LPS could promote homeostatic mechanism of B-1 B cells subset and B-1 cells appear first in ontogeny, after which B-1 cells decline in relative number over time as B-2 cell production proceeds. In adult mice, B-1 cells are the principal lymphocyte population in the peritoneal cavity and represent a small proportion (although possibly an equivalent number) of spleen B cells but are absent from lymph nodes and peripheral blood. A key unresolved question concerns the extent to which peritoneal and spleen B-1 cells represent the same population residing in different locations (Julia, 2006).

The aim of this study was to investigate, using *in vitro* systems, the response of stimulated and spontaneity, B-1 cells to entro-bacterial LPS.

MATERIALS AND METHODS

Animals: Male inbred Balb/c mice at 4-6 weeks of age were purchased from IRAN Pasteur institute. Animals were housed at least 1 week before experimentation in appropriate animal facility.

Sampling and tissue removal: The animals killed through the cervical dislocation regard to assistance from ethical notes on animal case committee. Blood collection take from heart and spleen were removed and cells were obtained by flushing and disintegrate by tissue grinder. Also peritoneal cavity cells harvested by gentle washing from another mice. (For each experiment led minimal used mice were 10). All the experiments and cells were collected from 10 mice and mixed together.

Isolation of spleen lymphocytes: Unseparated cells were obtained by spleen perfusion technique and then disruption by homogenized preparation with tissue grinder device to achieve maximum harvest from whole organ pieces. Suspensions of total spleen cells were washed and fractionate heterogeneous cell populations with centrifugation through ficoll-hypaque (1.09 Sigma

Hypauque) density gradient technique. After washing the interphase layer between ficoll and solution, the cell pellet resuspended for subsequent step ready for T cell depletion. For more separation of total lymphocyte, the preculture of spleen cells in complete tissue culture medium carried out according to remove adherent cells (peritoneal macrophages) for maximum purification of lymphocytes.

Isolation of lymphocyte from PBMNL: After Blood collection by heparinized syringe, haemodilution with phosphat buffer saline were carried out and separation of PBMNL by density gradient (Ficoll hypauque 1.09 zigma). The inter phase layer of PBMNL were collected and passed through the nylon wool column previously described to enrich the residual B lymphocyte.

Purification of lymphocyte from peritoneal lavage: Totally collected cells harvested from peritoneal lavage technique were washed with RPMI medium and slight RBC removed by 2 step lysing (0.16% hypertonic saline and then 0.2% hypotonic saline). The viable cells were incubated with CTM in tissue culture flask (25 mm^2) at 37°C humidified with 5% CO_2. The adherent cells (Mononuclear phagocytes) attached to the bed of culture after 4 h. The non adherent cells removed and washed 2 times with medium. The cells resuspended in CTM for subsequent reculture and stimulate with LPS. The purity of cells was checked by immuno fluorescent analysis.

Filtration of cells through nylon wool column: The column was prepared by packing 0.3 g of scrubbed combed ready for use nylon wool fiber (poly science Inc warrington.Pa) into 5 mL syring and autoclaving 15 min. After sterilization, the column was washed with RPMI and incubated at 37°C for 1 h. Then it was loaded with mononuclear spleen collected viable cells in a volume of 2 mL (10^7 mL^{-1}). The loaded column was incubated at 37°C for 1 h. The nylon wool adherent cells were collected by cold flashing 2 times after depletion of T cells. The collected cells were centrifugated at 250 g for 10 min. Cell pellet was resuspeneded in complete tissue culture medium and viable cells were counted. The purity of cells obtained after nylon wool purification was checked by IF analysis. When necessary, red blood cells were lysed in cell suspension with tris-ammonium chloride pH 7.2.

Stimulation of spleen and peritoneal B lymphocyte: Isolated and purified B cells from Blood, Peritoneal and spleen cells were cultured and stimulated with LPS (Zigma) in duplicated repeat in 24 well multiplate tissue culture dish. Finally cells incubated at 37°C humidified 5% CO_2 in various times including: 24, 36, 72 h. Unstimulated B cells as a control model for spontaneous secretion of IgM with any stimulus. The LPS dosage was specific for B cell stimulation (10 μg mL^{-1}).

Elisa for detection of secreted IgM: To measure immunoglobulin secretion by cultured B cell, supernatants were collected at the end of 24, 48, 72 h of cultivation the amount of secreted IgM and determine the total of IgM concentration in supernatant of cultured cells, by sandwich Elisa with Abs specific for murine immunoglobulin isotype (Mouse IgM ELISA Quantitation KIT [BETHYL] Cat E90-101, Lot E90-101-21). All 96-well trays were coated with 10 μg mL^{-1} isotype specific goat anti-mouse IgM polyclonal Abs for total IgM. Supernatant samples were added to the wells and the trays were incubated for 2 h. After washing an eluted solution in various stage with wash buffer containing Tween 20, Biotinglated mnAb against mouse IgM.

Stainings: The percentages of B lymphocyte was analyzed with double-staining IF technique cells at 2×10^5 suspended in 50 μL PBS were smear with cyto centrifuge air dried , fixed with cold (4°C) acetone and stained with the dyes. The phenotype was investigated with different combination of Abs. The expression of surface membrane CD3 and CD5 on isolated lymphocyte revealed the purity of B-1a cells.

Immuno fluorescent analysis: The enriched cell population was subjected to double staining florescence analysis to estimate the percentage of cells expressing C55, CD3 and B220 markers present on cells committed to B-1a ($CD5^+$ $CD3^-$) T cell ($CD5^+CD3^+$). Monoclonal Antibodies used for lymphocyte staining were included [PE conjugated Anti-mouse CD5 (ly-1), clone: 53-7.3, Cat#: 12-0051-81 (eBioscience) and FITC conjugated Anti-mouse CD3e, clone: 145-2C11, Cat#:11-0031-63 (eBioscience)]. Isotype matching as negative control with δ1 and δ2 conjugated PE and FITC were used to increase specificity of IF measurement. Each experiment was repeated at least three times and total results from all replicate experiments include analysis of each set of three or more independent experiments.

Cell viability: After Isolation and enrichment of harvested cells, viability was determined by trypan blue exclusion and counting cells with haemocytometer. Up to 90% viability has been confirmed.

MTT assay: Lymphocyte proliferation evaluation were performed on the basis of MTT assay and tested by:3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (ZIGMA).The proliferated and control cells freshly obtained and washed with medium by centrifugation (200 g, 5 min) and resuspend firmly for suspended cell preparations. Then plated in 96-well flat bottom tissue culture plate (100/000 cells per well) containing 10 μL of MTT solution (5 mg mL^{-1}) with 90 μL RPMI added per well. After 4 h of incubation in CO_2 5%, Formazan crystals were dissolved by 100 μL acidic isopropanol (ClH 4%) solution. Coloured plates after 5 min were read on a scanning spectrophotometer (Anthos 2020 Elisa reader) at 420 nm (measurement absorbance), 620 nm (reference absorbance) and calculate proliferation rate. Each samples repeated 3 times (triplicate) for Mean+/-SD determination according to maximum validation for evaluation.

Each experiment was repeated at least three times and total results from all replicate experiments include analysis of each set of three or more independent experiments.

RESULTS

To determine the effect of progressive *in vitro* stimulation of B-1a lymphocyte with appropriate specific dose of LPS (10 μg mL^{-1}) on poly specific IgM production, measured total concentration of IgM in supernatant of stimulated peritoneal, spleen and blood collected B1 lymphocyte by ELISA method is shown in Fig. 1. All the samples stimulated by LPS in IgM production show significant differences ($p<0.001$) comparison with control groups. In both of peritoneal and spleen IgM production differs from blood samples significantly ($p<0.05$). Twenty four hours cultured cells have significantly different between peritoneal and other samples in control groups.

Peritoneal and spleen B lymphocyte as a source of B1 cells and blood separated B lymphocyte as a B-2 conventional B lymphocyte. The first two at a time could be secreted IgM in lymphocyte response to appropriate minimal dosage of LPS and the later must be activated in different dose of

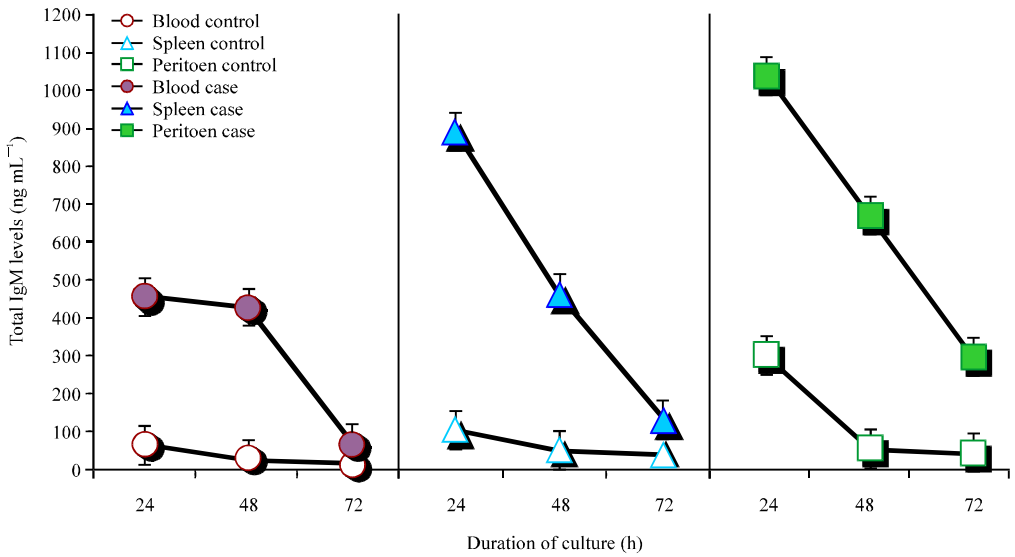

Fig. 1: Different concentration of detected IgM level from various source of B-1 cell collection based on duration of incubation time

Table 1: Result of lymphocyte proliferation assay by MTT method

		Ethanol+DMSO		
		24 h	48 h	72 h
Peritoneum	Control	0.159	0.1125	0.0225
	Case	0.258	0.1265	0.121
Spleen	Control	0.2792	0.2035	0.16
	Case	1.3395	0.268	0.190

LPS (maximum dosage). To explore the issue of some differences between spleen and peritoneal B1 lymphocyte in comparison with blood B-2 cells, functional characteristics of viability, Immunoglobulin production and stimulated proliferation was performed by MTT assay.

The results show that the peritoneal B-1 cells had significantly differ from case and control of spleen cells specially in 24 h ($p<0.001$). In 48 h culture of stimulated peritoneal B-1 cells, there were not any change and increase in proliferation and vitality activity. It is very surprising note that in 72 h culture of cells, very highly decrease in MTT index was happened. Also no significant differences have shown in case and control of peritoneal cells only mild in first 24 h of culture. Table 1 illustrate that the spleen B-1a cells proliferated more than peritoneal cells specially, before by the pass of time in comparison with spleen control and peritoneal B-1a cells. It shows that the spleen cells proliferated in high rate activity in 24 h but decreased in vitality performance after 48 and 72 h. Those response had significantly differ from control of unstimulated cells in 24 h ($p<0.001$). The peritoneal B-1 cells could not proliferated potentially *in vitro* but produced poly specific IgM in high concentration. On the contrary spleen cells had increase vitality and proliferation *in vitro* specially in 24 h but with lower level of IgM secretion (Table 1 and 2).

Data shows identified differences in surface marker expression of CD5 molecule by Immuno florescense analysis to evaluate this index marker as a main stimulus for potent secretion of IgM production. The expression of CD5 has been considered the principle phenotypic determinant in

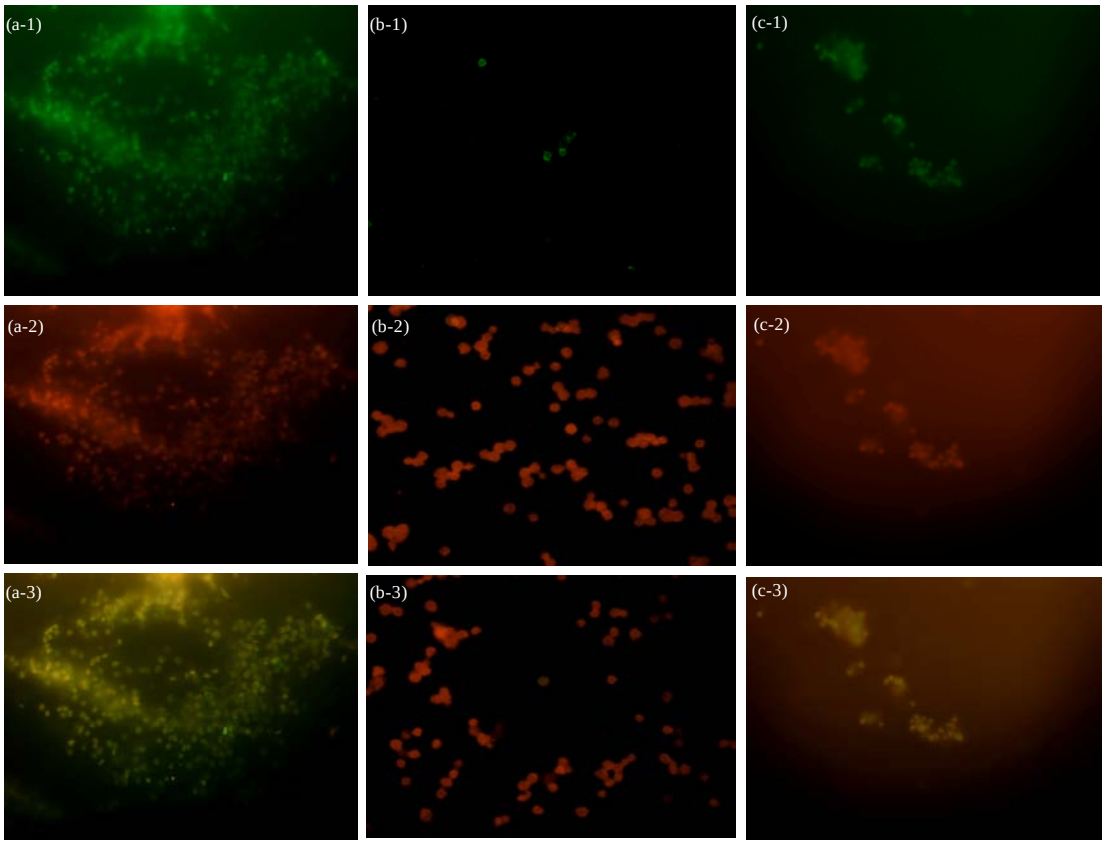

Fig. 2(a-c): Double staining imuno fluorescents imaging, (a) IF analysis of Spleen lymphocyte: (a-1 expression of $CD3^+$ cells, a-2 expression of $CD5^+$ cells, a-3 with co-expression of $CD5^+/CD3^+$ cells (T lymphocyte), (b) Isolated and purified B-1a cells from peritoneal lavages with maximum population of $CD5^+$ B cells: 1 expression of $CD3^+$ cells, b-2 expression of $CD5^+$ cells, b-3 co-expression of $CD3^-$ $CD5^+$ cells, (c) The harvested peripheral blood mononuclear leukocyte with different expression of CD3 and CD5 markers show that the lowest level of $CD5^+/CD3^-$ lymphocyte population (B-1a cells): c-1 expression of $CD3^+$ cells, c-2 expression of $CD5^+$ cells, c-3 co-expression of $CD3^+$ $CD5^+$ cells

Table 2: Expression of surface marker in different source of cells

	Peritoneum (%)	Spleen (%)	Blood (%)
$CD3^+/CD5^+$	5	40	60
$CD3^-/CD5^+$	90	30	5
$CD3^+/CD5^-$	<5	0	<5
$CD3^-/CD5^-$	0	30	30

defining these B cells as B-1 cell, regardless of location include peritoneal as a main place and spleen as a second milieu and Blood as a lacking site. Surface staining results for CD5 cells are shown in Fig. 2 peritoneal, spleen and Blood B-1 cells differed markedly in expression of surface $CD5^+$ (Table 2).

The vitality assessment of proliferated lymphocyte in cell culture system in various times could be seen in Table 1. It has shown that peritoneal cells were more prolonged cell survival and spontaneous IgM secretion in culture in comparison with spleen B-1 cells. Spleen B-1 cells vitality was reduced in comparison to peritoneal B-1 cells special was maintained during the culture period.

These data demonstrate both prolonged cell survival and spontaneous IgM secretion in culture are unique properties of peritoneal B-1 cells. The blood samples had effectual production from LPS stimulation.

DISCUSSION

B-1 cells with phenotypes and different functional specification form a particular population of B lymphocytes. These cells which homing in serosoal cavity attack against pathogens in contrast with specific IgM production by acquired immunity. B-1 cells can defend against foreign organism which attack body more than specific stimulation defense limit in poly specific form (Tumang *et al.*, 2004). In order to improve the validity of this research, the study design has performed by separated peritoneal B-1 cells and natural mouse spleen B cells by nylon wool column and nod adherent properties. Studies show that the main peritoneal lymphocyte populations are derived from B-1 cells. Although the spleen has B-1 cells in marginal region, it has other amounts of B-2 cells in lymphatic follicles (Tumang *et al.*, 2004; Hayakawa *et al.*, 1984).

The results of this study demonstrate that IgM antibody production against lipopolysacharide *in vitro* by peritoneal B lymphocytes had weak response against LPS stimulation in culture medium and finding confirms other related researches. Honjo *et al.* (2004) and Kawahara *et al.* (2003) have shown that B-1 populations in LPS 10-50 micrograms concentration are able to respond and produce antibody. But B-2 lymphocytes are able to response with 100 nanograms concentration (Julia, 2006). This finding was used well in this research because as there was not possible to separate spleen B-2 and B-1 cells, only spleen B-1 cells stimulated by high level concentration of LPS and actually, B-2 cells could not response to this concentration. Since spleen B-1 cells are weaker than peritoneal B-1 cells regarding to CD5 marker, so the superior ability of peritoneal B-1 cell in producing natural IgM is justifiable (Tumang *et al.*, 2004). Survival and function of spleen B-1 cell is just in case of stimulation with environmental condition like carbohydrate ligands and lypopolysaccharide (Wardemann *et al.*, 2002). They produced natural IgM against these antigens and multiply severely. But peritoneal B-1 cells become self renewed without LPS or any specific reactor and produce more IgM than spleen B-1 cells automatically (Tumang *et al.*, 2004; Boes *et al.*, 1998). This case hold true in our peritoneal samples.

Peritoneal B-1 lymphocytes stay active and alive in culture medium longer than B-2 population and in culturing duration, we observed their longer survival after 72 h and even 96 h.

Our findings were considerable relating to cells viability function and vitality function, although spontaneous production of IgM in control group was remarkable, too. The cells can continue producing before culture process and also can have considerable difference with stimulate samples.

In next 48 h, viability function and proliferation forces have decreased but are still regular and the obtained result show their viability function and vitality after 3 days which conforms Tumang *et al.* (2004) researches. Although, they used very specialized and accurate methods to prove this phenomena but the used MTT technique to prove peritoneal lymphocytes differentiate. The importance of having reactors was affirmed in 3rd day of culture for peritoneal cells because of vitality decrease. One of the significant points of observation was cells viability decrease or

affinity decrease in 48 h samples especially in case that IgM production happens nonstop in first 24 h. Feedback regulation effect is proved in scientists experiences. Observed result in spleen proliferation rate interfered by residual B-2 cells that not stimulated by those concentration.

It's possible to relate the high produced IgM concentration in spleen B cells culture to B-2 lymphocytes. It was not possible to separate B-1 and B-2 lymphocytes from gained cellular populations, because it needed accurate sorting system by using monoclonal antibodies by Magnetic Antibody Cell Sorter (MACS) method.

It was not seemed that the high IgM concentration in spleen cell culture is because of two phenomena: first of all B-2 cells in culture medium in response to Fetal Bovine Serum (FBS) of environment, are made to multiply and produce IgM. Although FBS material is essential to complete culture medium protein contents, but perhaps decreasing its concentration can put B-2 cells in least stimulation act (Tumang *et al*., 2004). In this case the necessity of using enriched culture medium without serum confirmed. The second is that although spleen B-1 lymphocytes were in touch with appropriate and standard LPS concentration (50 mg in culture medium per mL); but had less presence in comparison with peritoneal B-1 population. Our endeavor was to obey cell counting principles and we counted on cell response to LPS. It must be to consider that peritoneal B-1 cells had more ability to produce IgM although they had less viability than spleen lymphocytes. Researches show that IgM producing of peritoneal cells was high even in non reactor situation. In spite of spleen B lymphocytes impurity, IgM producing of spleen lymphocytes was not too much different from peritoneal lymphocytes. On the other sides of the research, studying spleen lymphocytes viability function, the reason of high relative viability function of spleen lymphocyte is the linked presence of both B-2 and B-1 group in spleen lymphocytes vitality function. As it is known spleen B-2 has more viability function than spleen B-1.

The main difficulty of this study was that impossibility to separate spleen B-1 and B-2 lymphocytes because it cost enormous expenses. But in fact there is also considerable difference in peritoneal and spleen B cells population.

Here, it is necessary to commemorate that producing spleen IgM by LPS reactor according to high dosage of LPS (10-50 μg mL^{-1}) is just for stimulating spleen B-1 cells (Julia, 2006). It was just able to find opportunity to stimulate spleen B-1 cells. If it may be to suppose to stimulate B-2 cells we had to decrease LPS dosage and were not able to produce IgM polysaccharide. High production of IgM against the LPS and LPS elution methods of biological products such as recombinant proteins which are always at risk of contamination with LPS derived from bacteria by plasmid vector are be enjoying.

In this study, due to complete depletion of cells and Antigen Presenting Cell (APC) (including macrophages, monocytes and dendritic cells) to LPS processing and activation of B-2 cells there. The same thing occurs in spleen B cells in marginal zone B-Lymphocyte.

MACS was not used in this study because recently have been demonstrated that cells passing through the MACS column in cell culture cannot survive and secrete cytokines and molecules such as antibodies and are not as an effective tool for lymphocyte purification while the use of nylon wool separation technique did not affect on cell activity, without pressure on cell vitality and ability to sustain the cell culture system.

CONCLUSION

This study concluded that the main source of B-1a cells is peritoneum cavity with high level of poly specific production of IgM. The spleen B-1 cells are very low in number; also peripheral blood mono nuclear cells have with lowest level of poly specific IgM provider cells. But *in vitro* culture of

those cells seem to be differing in IgM secretion. Spleen cells dominantly B-2 cell produce IgM against LPS in 24 h in culture system and then decrease dramatically in proliferation rate. But peritoneum B-1 lymphocytes with lower level of proliferation in comparison with spleen cells are potent provider of poly specific IgM. The peritoneal B-1 cells could not proliferate potentially *in vitro* but produce poly specific spontaneously IgM in high concentration.

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