**Isolation of B-1 subtype B lymphocytes from human cord blood**

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Abstract:

Introduction:
B-1 lymphocytes with high levels of CD5 markers are capable of differentiation and transformation into macrophage-like cells, as B-1 cells. This study aims to isolate and purify this unique subset from umbilical cord blood cell.

Materials and Methods:
14 umbilical cord samples from healthy neonates of natural or elective cesarean deliveries were used. White blood cells were separated from red blood cells using hydroxyethyl starch or gelatin 3%. Cell culture was performed to separate adhesive and non-adhesive cells. Umbilical cord lymphocytes subsets were separated from the remaining leukocyte contents by Ficoll density gradient centrifugation. Lymphocyte rosette test was used to assess purification stage.

Results:
Despite lack of difference in cell count between the two blood dilution methods, cell vitality in hydroxylethyl starch method was less than 87%, while cell vital activity in the dilution method by gelatin 3% was measured between 90 and 95%. Lymphocyte rosette test results indicate B-1 cell purification rate of approximately 99% by using the above method.

Conclusion:
This process indicates most of the extracted B-cells from human umbilical cord blood belong to the B-1 cell subtype, and gelatin 3% procedure is the best and most appropriate method in terms of number and vitality of cells.

Keywords: B lymphocytes, Blood, Macrophage

Introduction

Amazing discovery of B-1 cell derived from mononuclear phagocyte (BDMP) is one of the most controversial subjects associated with innate and non-specific immunity in recent years (1).

B-1 cells are a subset of B lymphocytes that develop in an independent status. These cells are created from blood-forming stem cells (derived from fetal liver). A large number of B-1 cells express CD5 molecule (2-4).

By studying phenotypic surface markers of the cells, a group of researchers found they were faced with dual-structured cells (5). These cells are the product of development, evolution, and beyond that, a heavy deformation that by migration to the peritoneal and pleural space in the rodent population, they can perform one of the major innate immune mechanisms in such
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a busy and important space. The pattern of this development is expected in human, but access to the path of development in this system and the final destination of migration, that is the peritoneal space, is not feasible. In performance, these cells are strongly phagocytic and they are capable in production and secretion of inflammatory factors and tissue exudates. They have the ability to perform cyclo-oxygenase-1 pathway and they are capable of responding to inflammatory and proinflammatory cytokines such as Gamma-interferon (6-7).

Presence of an extensive array of these cells in mice has been confirmed. In long-term cell culture studies, their macrophage-like activities have also been identified (8).

In adults, a large number of B-1 cells as a self-renewing population are found in the peritoneal and mucosal tissue. B-1 cells are created and spread faster than the qualified B cells. These cells express a modest treasure of the V genes and expose much less binding diversity than qualified B2 lymphocytes. B-1 cells spontaneously secrete IgM antibodies that often react with microbial polysaccharides and lipids. They also create a quick source of antibody production against microbes in specific locations, like the peritoneum. Studies show that in mucosa, these cells may differentiate into half of the IgA class of antibody secreting cells and they also contain limited antibody receptor deposits that generate early immune response (2, 6).

In 1989, Federico Caligaris et al. reported presence of significant amount of human lymphocyte B with CD5 marker in umbilical cord blood. Functional similarities of these lymphocytes with similar groups in mice samples have been demonstrated. Additionally, these cells are active in production of natural antibodies. B-1 cells can also be proliferated and in special circumstances, they can migrate to peritoneal and thoracic cavities and provide a large proportion of the natural defenses. Interleukins 4 (IL-4) and cytokines regulation of B-cells growth are involved in these cells activation process (1, 3, and 9). Research about B-1 cells function shows that message transmission plays an important role in their development. These cells are unable or not involved in the formation of reproductive center. Also, it has been found that in terms of antibody production, peritoneal B-1 cells are dormant, but in the spleen, they are responsible for producing over 50% of antibody-secreting cells (10, 11).

Being in micro-environments that contain certain cytokines moderately changes appearance and functional properties of the cells, but there is little information about the changes in the environments other than body cavities (12).

Studies indicate that human umbilical cord blood contains adhesive precursor cells, comparatively less than bone marrow, which may be distinct in different stages (13). The present study was designed despite inaccessibility of these cells in human, aiming to utilize umbilical cord blood cells from healthy neonates born of healthy and young mothers as a source for development of B-1 cells in a conventional cell culture medium. The main objective was isolation and purification of B-1 cells from a set of human umbilical cord blood lymphocytes.

Materials and Methods

In this experimental study, 14 umbilical cord samples from healthy neonates of natural or elective cesarean deliveries were used by random sampling. Umbilical cord blood was collected in the following two standard methods:

A) Use of a special umbilical cord kit, containing: blood collection bag, calibrated tube, and flask and ice pack. All blood sampling stages were carried out in the delivery room under sterile conditions by the staff at the umbilical cord blood bank affiliated to the Royan Institute. The entire set was transferred to the laboratory by maintaining standard conditions at 22±4 °C.
B) Collection of umbilical cord blood from neonates born by cesarean section in the operating theater. To this end, special blood sampling tubes had been coated by 50 IU/ml of heparin for every milliliter of blood. Then, the cord content was slowly emptied by leading the cut end of cord toward the tube. The above stages were carried out in the cesarean room at Parsian hospital. It should be explained that subjects were selected for the study after obtaining full consents and performing health-related tests including HCV, HBV, HIV, and syphilis on umbilical cord blood donating mothers. Exclusion criteria were the positive result of HIV, HBV, HCV and syphilis test in mothers.

**Separation and elimination of red blood cells from umbilical cord blood contents:** In order to better isolate white blood cells and avoid presence of fetal red blood cells in umbilical cord blood, the following two methods were used:

C) Use of hydroxyethyl starch: The density gradient of hydroxyethyl starch diluted to 10% with PBS was used. This density gradient is capable of depositing majority of umbilical cord red blood cells, especially those with nucleus. Blood samples were mixed with hydroxylethyl starch in a 1 to 5 ratio. After incubation for an hour at room temperature, leukocyte-rich plasma was extracted from the contents of the upper section of tube by a plastic suction and Pasteur pipette. This collection contains the total leukocytes in the umbilical cord blood and after counting and verifying their cell vitality rate, it was centrifuged at 250 rpm for 10 minutes and kept for the next stage; that is, isolation of mononeuclears.

D) Use of gelatin 3%: In this method, blood samples were mixed with a solution of gelatin 3% in 0.9% PBS with volume ratio of 1 to 1 and incubated at room temperature under a laminar hood. Red blood cells which are young and nucleated, have a high specific gravity and pass through gelatin cross-gradient, while heavy red blood cells are precipitated due to the gelatin-assisted increased gravitational force. After removing surface contents, centrifuge is performed at 400 rpm for 10 minutes. Process of purification continued with cell count and determination of vitality rate. At this stage, vitality rate should not be less than 95%.

**Isolation and purification of non-adhesive umbilical cord blood cells:** Stage of cell culture is used for isolation of adhesive cells such as: fibroblastoids, mesenchymal stem cells, vascular endothelial cells, and monocytes found in umbilical cord blood circulation and other adhesive cells. Incubation took 3 days at 37 °C, CO2 pressure of 5%, and saturated humidity. During this time, monocytes adhered to the bottom of flask and in this way, adhesive cells were isolated from non-adhesive cells. Degree of purity and cell vitality (over 90%) were determined with cell count and trypan blue staining. Finally, non-adhesive cells were centrifuged at 250 rpm for 10 minutes in the same culture medium (4).

**Separation of umbilical cord blood lymphocyte subsets from residual leukocyte contents:** Culture supernatant from previous stage of culture, containing umbilical cord blood mononuclear cells and granulocytes were re-suspended with appropriate volume. Initially, separation of cellular subsets was performed using Ficoll density gradient (molecular weight=1.077). During the 30-minute centrifuge at 400 rpm at 4°C, mononuclear cell layer was formed on the upper part of Ficoll layer and lower region of the diluted plasma. This cellular layer was carefully removed with a plastic suction and Pasteur pipette. This layer contains T lymphocytes, B lymphocytes (mostly B-1 group), and NK cells. Contents were mixed again and centrifuged at 250 rpm at 4°C for 10 minutes. It should be noted that in this separation method, presence of some umbilical cord red blood cells is possible. Still, mononuclear cell purity in the above set should not be less than 70% (14).
Isolation and purification of B lymphocytes from umbilical cord blood mononuclear cells:

Cell count and vitality rate were determined after collecting umbilical cord blood mononuclear cells amid the Ficoll and the lower region of plasma. To access B lymphocyte cells, nylon wool columns (prepared from blood transfusion set) were used. Appropriate amount of cell suspension was poured in culture medium using Pasteur pipette in the columns. Filled columns with cells were incubated at 37 °C (preferably water bath). Then, by placing into the Falcon tube mouthpiece and opening the column outlet (previously blocked with parafilm) contents of columns were removed. For full discharge of non-adhesive cells, upper mouthpiece of the column was freed. Initially, nylon wool columns were washed three times successively by maintaining previous temperature. B cells containing some lectin receptors, bind to nylon wool and do not discharge from columns in the first stage. For their discharge, cold shock by cooled RPMI medium is required (2, 15). To remove B lymphocytes, cold shock was performed using cold culture medium (4 °C) and washed twice. Then cells were centrifuged at 250 rpm at 4 °C for 10 minutes. To assess purification stage, sheep red blood cells were used for the lymphocyte rosette test (16, 17).

Results

The aim of this study was to determine an efficient and reliable method for purification and isolation of B-1 lymphocytes from umbilical cord blood lymphocyte subsets. Purification of white blood cells by performing two isolation techniques (use of hydroxyethyl starch, and gelatin) showed that dilution of blood with hydroxyethyl starch gradient, despite highly rapid precipitation of umbilical cord red blood cells, caused loss of cell vitality. Thus in this study, gelatin technique was preferred (table 1).

Despite the lack of difference in cell count between the two blood dilution materials, cell vitality in hydroxyethyl starch method was less than 87%, while in the gelatin 3% dilution method was measured between 90 and 95%. The two methods of blood collection (use of blood bag and leading tube by heparin) did not influence the results in this stage. Observations revealed 3 days incubation and cell culture in sterile condition in special cell culture flasks enabled almost full removal of primary adhesive cells (figure 1). Approximately 90% of cells were removed in this culture stage.

Separation of lymphocytes in cell suspension from leukocyte myeloid lineage and hematopoietic stem cells (probably associated with this lineage) was performed using Ficoll gradient. Results indicate that despite heavy load of red blood cells in associate with leukocytes, a major part of purification had been achieved (table 1). Fortunately, the least loss of cell vitality was observed in this stage. However, in the cell proliferation study, up to 10% of red blood cells still existed. Lymphocyte rosette test using sheep erythrocite showed mean 70% of nylon wool exit cells were positive, which is indicative of accuracy in the procedure and T lymphocytes isolation (figure 2). Lymphocyte rosette test result was 99% negative for cells removed from nylon wool by cold shock.

Discussion

Biology and evolution scientists’ studies have shown that despite the functional differences in specific immunity and innate defense mechanisms, in many instances, not only sources and evolutionary roots of these two systems are similar, but also, they are derived from the same patterns. It has been proven that main cells in these two systems have common ancestry, but the interesting point is the ability of cell components in these systems to develop and transform into each other. One of the best examples of such a
development is the B lymphocyte population, especially the B-1 subset. Despite exhibiting the extraordinary features in development of both innate and specific defense systems, these cells have the ability of ultra-embryonic evolution during blood and tissue development. One of these phenomena is the ability to transform into a sub-type called B-1b cells. The particular figure of this cell and its structural and functional developments add to its complexity and despite understanding of a lot of effective mechanisms in continuation these developments in rodents; there are still vague points about evolutionary and development path of this subsets in human (16).

Morphological developments of B-1 cells in rodents have been fully proven. The origin of sampling and addressing to functional nature of this cell is the peritoneal region in mice. According to studies, phagocytic capabilities of these cells have been recognized (4, 7-8, 17-18). In humans, access to this lymphocyte population is almost impossible. Despite understanding similar mechanisms in humans, there are still ambiguities in the way these cells develop. Healthy infants’ umbilical cord blood is one of the most important and a unique source of these cells, but their number is very limited. A major part of umbilical cord blood B lymphocyte population is B-1 type (about 90%) (19, 22).

Healthy neonates’ umbilical cord blood has found many applications in modern medicine and is used as a significant source of hematopoietic precursor in treatment of a variety of congenital disorders and malignant diseases. One of the most important reasons for umbilical cord preference over bone marrow is less incidence of immunopathological transplant rejections. Although extraction and preservation of B-1 cells from umbilical cord is easy, they have lesser cell content than bone marrow. There are many different techniques for reinforcement and proliferation of umbilical cord blood cells. So with their help, target cell population can be strengthened and sufficiently utilized (19, 20).

Majority of researchers that have studied and compared stem cells derived from umbilical cord blood, bone marrow, and lipid tissue, ultimately recommended umbilical cord blood as an ideal source for therapeutic purposes (21, 22). Although objectives of most cellular and molecular studies on umbilical cord blood are associated with its therapeutic aspects, addressing the function of immune qualified cells in this subset has less been considered. As the differentiation rate of stem cells tends increasingly toward hematopoietic lineage, the exposing risk of this compatibility indicator and transplant rejection is increased (21).

The present study showed heterogeneity in lymphocyte subsets count in different samples. Yet, different umbilical cord blood samples are different in number of cells, particularly B-1 lymphocyte population which was higher than those suggested in references and articles (21, 22).

Some sources use special red blood cell lysis buffer or distilled water with successive concentrations 0.2% and 1.6% to remove this amount of red blood cells. According to previous experiences, these techniques cause loss of cell vitality. In the present study, attempts were made to remove red blood cells possible by accurate aspiration of mononuclear cells layer on the Ficoll. Yet, purification rate of mononuclear cells in the above set should not be less than 70% (13, 20).

On the other hand, since B cells still contain some lectin receptors and bind to the nylon wool, they cannot be removed from nylon wool column in the first stage and cold shock by culture medium or cooled RPMI is required for their removal. Lectin receptors of these cells are very high in numbers and strongly bind to the nylon wool and cannot easily be removed by cold shock (2, 15).
Table 1: Cell count in different stages of isolation and culture

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Sample volume</th>
<th>Primary cell count after removing red blood cells using gelatin and hydroxy-methyl starch</th>
<th>The number of mononuclear cells after removing adhesive cells</th>
<th>Mononuclear cells after Ficoll gradient</th>
<th>The number of B lymphocytes after nylon wool</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15ml</td>
<td>15.1*10^6 gelatin</td>
<td>6.5*10^6</td>
<td>4*10^6</td>
<td>3.2*10^6</td>
</tr>
<tr>
<td>4</td>
<td>50ml</td>
<td>17.7*10^6 Gelatin</td>
<td>6.1*10^6</td>
<td>5.2*10^6</td>
<td>4.7*10^6</td>
</tr>
<tr>
<td>5</td>
<td>50ml</td>
<td>27*10^6</td>
<td>10*10^6</td>
<td>7*10^6</td>
<td>5.45*10^6</td>
</tr>
<tr>
<td>6</td>
<td>49ml</td>
<td>18*10^6 Gelatin</td>
<td>7*10^6</td>
<td>5*10^6</td>
<td>4.25*10^6</td>
</tr>
<tr>
<td>7</td>
<td>5ml</td>
<td>17.86*10^6 Gelatin</td>
<td>4*10^6</td>
<td>1.5*10^6</td>
<td>750*10^6</td>
</tr>
<tr>
<td>8</td>
<td>35ml</td>
<td>82.5*10^6 Hydroxy-ethyl starch</td>
<td>35*10^6</td>
<td>19.5*10^6</td>
<td>8.5*10^6</td>
</tr>
<tr>
<td>9</td>
<td>16ml</td>
<td>29*10^6 Gelatin</td>
<td>15.78*10^6</td>
<td>7.5*10^6</td>
<td>10.04*10^6</td>
</tr>
<tr>
<td>11</td>
<td>25ml</td>
<td>144*10^6 Hydroxy-ethyl starch</td>
<td>61.6*10^6</td>
<td>33.76*10^6</td>
<td>12.2*10^6</td>
</tr>
<tr>
<td>12</td>
<td>22ml</td>
<td>75.32*10^6 Gelatin</td>
<td>65*10^6</td>
<td>31.6*10^6</td>
<td>21.6*10^6</td>
</tr>
<tr>
<td>13</td>
<td>8ml</td>
<td>22.4*10^6 Gelatin</td>
<td>11.3*10^6</td>
<td>8*10^6</td>
<td>1.6*10^6</td>
</tr>
<tr>
<td>14</td>
<td>20ml</td>
<td>140*10^6 Hydroxy-ethyl starch</td>
<td>97.5*10^6</td>
<td>35*10^6</td>
<td>24*10^6</td>
</tr>
</tbody>
</table>

Figure 1: Modified lymphocyte rosette to increase accuracy of lymphocytes resulting from removal stage. The B cells do not exhibit any reaction with sheep red blood cells (s-RBC). Majority of cells are negative in terms of rosette formation (Magnification x40).
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In this study, two methods of percoll and nylon wool were used for the purification of B lymphocytes with density gradient. In the percoll method, fetal red blood cell resistance leads to agglomeration and lack of purification in lymphocytes, so that very few lymphocytes can pass through this stage and thereby reduces possibility of purification of B lymphocytes, which are a small population of total lymphocytes. While despite being older, nylon wool technique is sufficiently efficient for separation of B cells and T cells from human umbilical cord blood. Cell count after this stage is witness to efficiency of this method. In this study, efforts were made to introduce an optimal and efficient method of extraction and isolation of B-1 lymphocytes from human umbilical cord blood by maintaining cell vitality and morphology. Results from different stages of this experimental process clearly showed successful extraction and purification of this cell from a human source. B-1 cells that finally removed from nylon wool were proven through the rosette test. In fact stem cells are from the B1 subset with a wide range of possibilities in therapeutic and research applications.

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References: