MORPHOLOGICAL AND CYTOCHEMICAL CHARACTERISTICS OF PURIFIED MURINE SPLENIC DENDRITIC CELLS

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ABSTRACT

Dendritic cells function as the main cellular population responsible for professional antigen presentation and hence for induction of primary immune responses. Although they are present in virtually every tissue, nevertheless their number is usually so low that it makes their isolation for studies very difficult.

In this study, we purified dendritic cells from mouse spleen by a three-step enrichment method and evaluated morphological and cytochemical characteristics of isolated cells.

We showed that isolated dendritic cells from mouse spleen had all lobulated nuclei with multiple cytoplasmic projections and their morphological features changed after an overnight incubation. It was also shown that typical dendritic cells lacked both Myeloperoxidase (MPO) and Non Specific Esterase (NSE) activity.

In conclusion, for reaching a reasonable purity in isolation of dendritic cells from lymphoid tissues, many enrichment steps should be taken, and for determining the purity of isolated cells, we recommend that a combination of morphological and cytochemical studies be used.

Keywords: Dendritic cell, Cytochemistry, Morphology, Myeloperoxidase, Nonspecific Esterase

INTRODUCTION

Dendritic cells (DC) were first described in 1973 by Steinman and coworkers in lymphoid organs of mouse.[1] DCs are among professional antigen presenting cells with a unique ability to induce primary immune responses. Precursors of DCs root from bone marrow and home in peripheral tissues after circulating in blood.[2] During infections, DCs take up antigens of invading microoganisms and migrate to T cell areas of secondary lymphoid organs through efferent lymph nodes.[2-4] It is in these organs that DCs present antigens to specific $CD4^+$ T cells resulting in their activation and hence provoking primary immune responses. $CD4^+$ T cells, in turn, activate and regulate the response of other cells of immune system such as B cells, CD8+ T cell's NK cell and macrophages. Therefore, DCs play a central role in the stage of both humoral and cellular immune responses.[2] Other than lymphoid organs like spleen, lymph nodes and thymus, dendritic cells are found in non-lymphoid tissues such as skin, respiratory tract, alimentary tract, liver, pancreas, eye, central nervous system and organs of reproductive system.[5]

Like any other cell population, in-vitro evaluation of DC functions such as induction of cytokine produc

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tion, antigen presentation and T cell activation as well as histochemical and ultrastructural studies of these cells require purified cell population. One problem that especially makes studies on DCs difficult is their scarcity in different tissues; as an example, less than 0.5% of all nucleated cells in monouclear suspension of mouse spleen is composed of dendritic cells.[1,6]

We, therefore, present here a three step procedure for obtaining high yield and purity of dendritic cells from mouse spleen. In addition, the morphological and cytochemical characteristics of the purified DCs will be presented.

MATERIALS AND METHODS

A) Animals:

Male Balb/c mice (8-12 weeks) were purchased from Pasteur Institute of Iran. Mice were kept under optimal conditions of hygiene, temperature, humidity and light (cycles of 12hr dark / light). All experimental procedures on animals were approved by the ethical committee of Avesina Research Center.

B) Purification of splenic dendritic cells:

Low-density cells from mouse spleen were prepared as previously described.[7] Briefly, spleens were removed under sterile condition and each spleen was then injected with a cocktail comprising of 0.5 mg/mL collagnase D(Roche Diagnostic, Mannheim, Germany) and 30μg/mL DNase I(Roch Diagnostics, Mannheim, Germany). The resultant cell suspension was kept on ice. Collagenase D (1.2mg/mL) and DNase I(30μg/mL) were added to the spleens after they were minced into very small pieces and incubated at 37°C for 40 minutes. EDTA with final concentration of 5mM was then added and the tissue fragments were pipetted several times. The dissociated tissue was sieved through metal mesh and the obtained cell suspension was mixed with the suspension obtained from collagen injection. Total suspension was washed twice with PBS containing 5mM EDTA each for 10^{min} at 4°C, 280×g. Cell pellet was immediately mixed with 15% Optiprep (Axis-Shield PoC AS, Oslo, Norway) with the density of 1.85g/mL. Optiprep 12% and CMF HBSS (Ca^{2+}, Mg^{2+} Free Hanks Balanced Salt Solution) were consecutively and slowly overlayed. Tubes were centrifuged 600g at 20°C for 15 minutes. Low density (LD) cells were removed from the interface of CMF HBSS and 12% Optiprep, washed twice with cold PBS-EDTA at 4°C. Pellet was washed with RPMI-1640 (Sigma, St Louis, USA) and cultured in 60×15mm Petri dishes (Falcon, NY, USA) in a CO_2 incubator with 5% CO_2 at 37°C for 2 hours. Non adherent cells were then discarded by several washings with warm (37°C) RPMI-1640 containing 5% fetal calf serum (Invitrogen, Eggenstein, Germany). Warm RPMI was added to the adherent cells and plates were incubated with 5% CO2 at 37°C for 14-20 hours, after which the floating DCs were removed and washed twice with cold PBS at 4°C. The obtained cells were cytospinned onto poly-L-Lysine coated slides.

C) Purification of PMN from human blood:

EDTA-treated whole blood was centrifuged at 200g for 15-20 minutes at 18-22°C. The buffy coat was harvested in a 1ml volume of plasma supernatant. Leukocyte – rich plasma was then layered under 1.077 g/mL and the same volume of 1.090 g/mL solutions of Optiprep (Axis-shield Poc AS, Oslo, Norway) and centrifuged 800g at RT (Room Temperature) for 25 minutes. Granulocytes were removed from the lower interface. The cell suspension was then washed with a diluent solution (0.85% Nacl, 1 mM EDTA, 20 mM HEPES, PH= 7.4) and contaminating erythrocytes were lysed by NH4Cl (0.83%, PH= 7.4) buffer. The purified PMNs were later used as positive controls for MPO activity.

D) Cytochemical staining of cells:

For detection of MPO activity, slides prepared by cytospin were immersed in incubation mixture (DAB,5 mg; Tris-HCl; 0.05M pH= 7.6 10mL; H_2O_2, 30% 0.1ml) for 5 minutes and then washed with Tris-HCl buffer. Slides were then immersed in reaction enhancer solution (0.5% $CuSo_4$ in Tris-HCl buffer). After being washed, slides were counterstained with Gimsa, dried and mounted.

Activity of non-specific esterase (NSE) was revealed by incubation with a mixture of hexazotized pararosaniline and alpha naphtyl acetate (Merck, Germany) (pH= 6.3) as the substrate for the enzyme for 40 minutes at RT. Slides were then washed, counterstained in hematoxyline, cleared and mounted.

E) Immunocytochemical Staining:

Cytospinned cells were fixed for 2 minutes in cold acetone and washed three times with PBS. The endogenous peroxidase was neutralized by H_2O_2, 0.3% in Tris Buffer Saline 0.15M, PH= 7.4. Biotin-conjugated anti IA/IE antibody (Pharmingen, SanDiego, USA) was then added and slides were incubated at RT for 1 hour, followed by three times PBS wash. Slides were then incubated with 1:200 HRP-Strepavidin (Biosource International, CA, USA) for 30 minutes. Excess antibodies were washed off with three PBS washes followed by addition of Diaminobenzidine, DAB(Roche Diagnostics, Mannheim, Germany) solution. After color development, the reaction was stopped by washing the slides under tap water. Slides were then dehydrated by graded doses of alcohol and mounted after counterstaining with hematoxylline.

Staining with anti-CD11c (pharmingen, SanDiego, USA) differed from the above procedure in that 4°C slides were incubated overnight at 4°C after addition of antibody. Following three washes with PBS, biotin conjugated hamster anti-mouse immunoglobulin containing 1% normal mouse serum was added and incubated for 1 hour at RT. The staining was completed as above.

RESULTS

A) Purification of splenic DCs:

Each mouse spleen yielded about 200-300×10^6 (mean= 255×10^6) leukocytes after enzymatic digestion with collagenase and DNase.

We used Optiprep gradient medium with the density of 1.068 for first step enrichment of spleen DCs and the yield of LD cells in such density was 3-6% of all spleen cells.

Phase contrast microscopic analysis of LD cell cultures revealed that just after 15 minutes DCs adhered to the bottom of plates and spread out slowly. Due to a high number of cultured cells in each plate, the exact characterization of dendric cells including the recognition of their cytoplasmic projections was not easily possible at this stage.

After approximately 1.5-2 hours, DCs adhered firmly and spread their distinctive projections. Other cells which remained nonadherent composed mostly of lymphocytes, some macrophages and a small group of DCs, and were easily removed by several pipettings. Removal of non-adherent cells made the exact morphology of DCs recognizable by invert microscopy. At this stage, only 0.4% of all spleen cells remained adherent to the plate.

Phase contrast microscopy showed that these irregularly shaped cells had multiple processes extending from an oval cell body (Fig 1). These processes appeared in variable shapes, from small swellings to large humps and to very long and thin spindle like projections. Each Dendritic cell had 1 to 7 projections on its surface. Some minor branches were seen budding from main cytoplasmic projections. DCs were mainly observed under phase contrast Microscope as dark and satellite-like cells. The majority of dendritic cells became mature after 12-16 hours of culture which allowed them to float, while most other cell populations including B lymphocytes and macrophages remained attached to the plate. A group of DCs even formed small or large aggregates in suspension. Overnight culture resulting in maturation of DCs caused their projections to become longer and more in number but less in width. Gimsa staining of DCs revealed their bean-shaped or biconvex nuclei, and some, but not all, of their cytoplasmic projection. Overall, the yield of purified DCs from each mouse spleen was 5-7×10^5 cells.

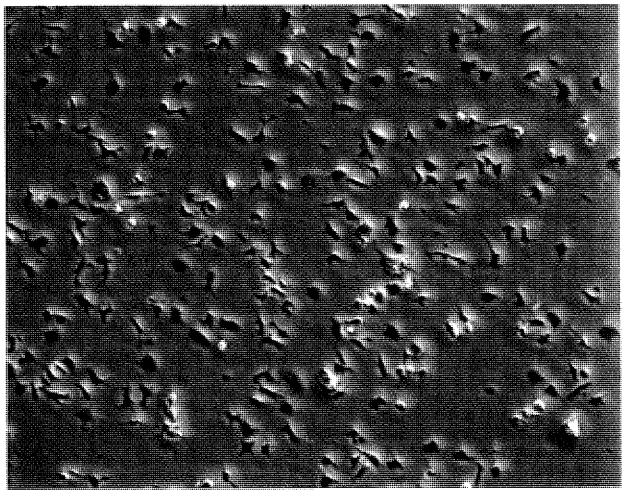

Fig. 1. Characteristic morphology of mouse spleen dendritic cells under phase contrast microscope (100x).

B) Immunocytochemistry:

Purified DCs were cytospined on poly L-lysine coated slides. Two markers, MHCII and CD11c, were used to evaluate the purity of isolated cells. Expression of CD11c was observed on more than 97% of the cells and on nearly all cell with morphological characteristics of dendritic cells (Fig 2). MHC II was also present with even higher density than CD11c on all the morphologically identified dendritic cells (Fig 3).The purity was also checked by flowcytometry using the same antibody and FITC conjugated mouse anti-hamster and found to be approximately 94%.

C) Activity of NSE and MPO:

The activity of the two enzymes, NSE and MPO, was assessed in purified spleen DCs. Mouse perito-

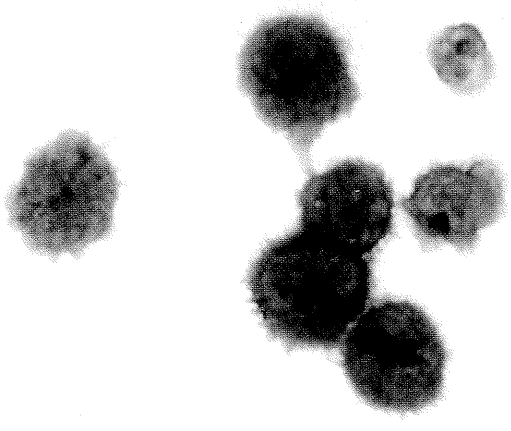

Fig. 2. CD11c+ purified dendritic cells.

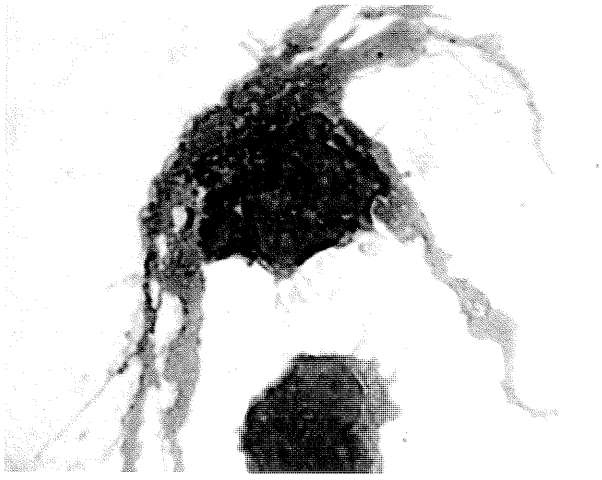

Fig. 3. A typical dendritic cell stained with anti MHC II antibody.

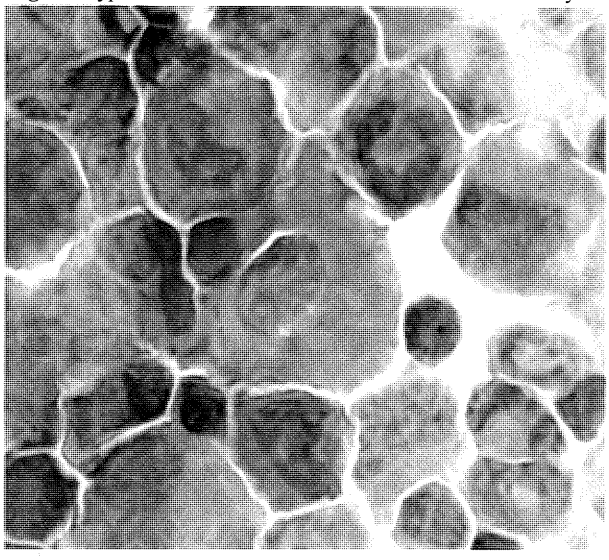

Fig. 4. Cytochemical staining of peritoneal macrophages showing high reactivity for NSE.

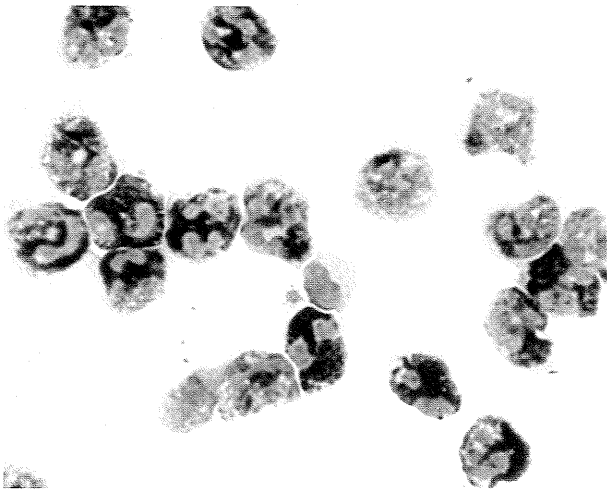

Fig. 5. Cytochemical staining of Human peripheral blood PMN showing high reactivity for MPO.

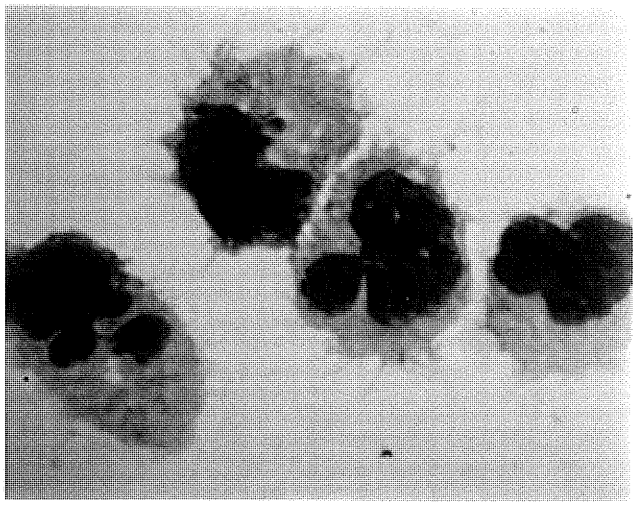

Fig. 6. Cytochemical staining of purified mouse spleen dendritic cells with no activity for MPO.

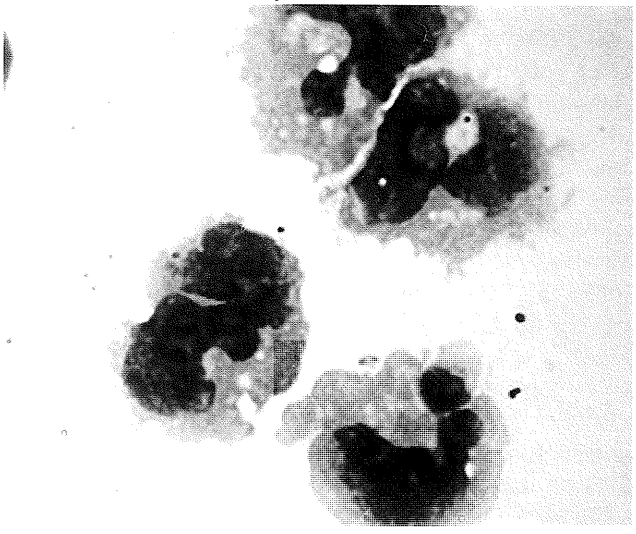

Fig. 7. Cytochemical staining of purified mouse spleen dendritic cells with no activity for NSE.

neal macrophages were used as positive control for NSE activity and human peripheral polymorphonuclear cells for MPO activity. Mouse peritoneal macrophages were highly positive for NSE (Fig 4). Human PMNs also showed remarkable activity of MPO activity (Fig 5). Murine spleen DCs lacked both MPO and NSE activity (Fig 6 and 7, respecively).

DISCUSSION

We presented here the morphological and cytochemical characteristics of murine splenic DCs. No cell line has been introduced so far for DCs, therefore it is required that these cells be freshly prepared from tissues for any investigation involving dendritic cells. One major problem in such studies is the scarcity of DCs in most tissues, for instance dendritic cells com-

prise less than 0.5% of all murine splenic leukocytes.[6] This extremely low frequency of DCs makes it impossible to isolate these cells using one-step purification method with reasonable purity and efficiency. Thus only combination of different purification methods may result in isolation of DCs with acceptable density and purity for most cytological studies.

In most isolation methods, distinctive features of dendritic cells such as low density, rapid adherence to plastic and glass and floatation after overnight culture are utilized. We also used these characteristics to isolate dendritic cells from mouse spleen. In the first step, DCs were separated from high density cells since only 3-6% of all spleen cells remain bouyant at this density. This step enriches DCs for about 20-30 times. In the second step, we took advantage of the ability of DCs to firmly adhere to glass and plastic surfaces. Immature mouse and human DCs, like macrophages, have a tendancy to stick to glass and plastic surfaces, nylon wool and sepharose.[8,9] This step allowed the nonadherent cells which comprised of the majority of contaminating cells to be successfully removed. In the last step of our procedure, the unique feature of losing their ability to bind to surfaces upon maturation was employed for isolating DCs from other adherent cells such as macrophages. Macrophages start sticking to the bottom of culture plate 1 hour after culture and maintain their adherence for several days, and are quite different from DCs which mostly float after overnight culture.[8]

In our cytochemical studies, we observed that murine splenic DCs were MPO^-. Other investigations indicated that human peripheral DCs lack this enzyme[11-12] and more interesting, even DC precursors in bone marrow have been shown not to have any detectable activity of this enzyme.[13] Although there are reports of MPO^+ human DCs, these are restricted to the subtypes that were generated in-vitro under the action of IL-4 and GM-CSF from monocytes. Otherwise, human DCs originated from bone marrow precursor were all MPO^-.[14] MPO has an important role in breaking H_20_2 and producing toxic components. Hydrogen peroxide is a well known germicidal agent whose activity is greatly enhanced by peroxides and halides. The interaction with chloride ion for example, produces hypochlorous acid. The reaction is catalyzed by Myeloperoxidase.[15] This system has been shown to be highly reactive and toxic to a variety of microorganisms and tumor cells. As for cellular distribution of MPO, it is present primarily in the primary granules of resting neutrophils. Monocytes contain MPO^+ granules, but macrophages lose their granular peroxidase during maturation in tissues.[15] Although immature DCs are able to phagocytose foreign materials, they lose their ability as they mature, and take on another function in adaptive immune responses which is the critical step of antigen presentation to T lymphocytes. Therefore, it is easily conceivable that dendritic cells lack MPO activity as was shown in our study because their function dictates quite different destiny from tissue scavengers such as neutrophils or monocytes.

We also showed in our study that murine splenic DCs were negative for NSE. There are extensive and somehow contradictory data available about the expression of NSE in DCs of different organs and different species. For example, it was shown that human peripheral DCs,[10,12] rat lymph node DCs[16] and rat liver DCs[17] were NSE^-, whereas the Langerhance cells (LC) as a subgroup of dendritic cell family were demonstrated to be NSE+.[18,19] Thus DCs are heterogenous regarding the expression of NSE.

Intestinal lymph nodes of rat contain two distinct subgroups of DCs. A group of these cells that are strong inducers of immune responses lack NSE, while those that have poor antigen presenting capability show NSE activity. The latter subgroup are believed to take in debris from apoptotic epithelial cells and migrate to T cell areas of local lymph nodes. Their main function is thought to be the induction and maintainance of peripheral tolerance.[20]

There are few reports on the biological role of NSE. Recent data showed that administration of NSE inhibitor (Bis 4 Nitrophenyl phosphate BNPP) interfered with macrophage migration and reduced their phagocytic activity.[14] It, therefore, seems possible that NSE is somehow involved in chemotaxis and phagocytosis of phagocytic cells.

By integrating these data, it seems probable that NSE^+ DCs phagocytose and transfer foreign antigens to local draining lymph nodes. Then, efficient antigen presenting DCs which are mostly NSE^- present the antigenic epitopes through a process known as crosspresentation.[21]

Since most of the contaminating cells in the majority of DC purification protocols are macrophages and regarding the fact that monocyte and macrophages are NSE^+ while DCs are NSE^-, we recommend to use this test for assessment of the purity of isolated DCs. In a recent investigation, Karhumaki and coworkers successfully used NSE test for determining purity of isolated DCs from human peripheral blood.[12] In our study, DCs were isolated from murine spleen with the purity of 97% determined by anti CD11c which has been reported as a specific marker for murine DCs,[22] More than 95% of those isolated cells were NSE^-.

There is evidence for at least two separate developmental pathways for murine DCs namely, lymphoid and myeloid pathways. Myeloid and lymphoid dendritic

cells differ remarkably regarding their phenotypes, localization and function. They although are similar in many aspects; both groups highly express CD11c, MHCII, CD86 and CD40. Myeloid DCs express in addition to the above molecules, specific marker of CD11b, whereas lymphoid ones are $CD11b^{-}$ and instead have other markers such as DEC-205 and $CD8^{2}$. DC specific markers of MHCII and CD11c were employed in our study to evaluate immunophenotypical features of isolated DCs. Characteristic appearance of dendritic cells together with CD11c expression were used for determination of purity of isolated murine DCs. By using these criteria, we showed that our isolated dendrite cells had a purity of more than 97%.

Dendritic cells have unique morphological characteristics and specific culture behavior. These features together with their lack of reactivity of MPO and NSE make it possible to accurately and yet conveniently assess the purity of isoated dendritic cells from mouse spleen.

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