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A Two Step Procedure to Fractionate Mouse Testicular Macrophages with Different Cytokine Profiles

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Abstract. Cells isolated enzymatically from the interstitial tissue of mouse male gonads are composed of macrophages, Leydig cells, and myofibroblasts. They can be separated on density gradients, either by sedimentation (Ficoll) or flotation (Percoll) into several fractions according to the different buoyant densities of the different cells in the mixture. Macrophages ($Fc\gamma R^+$, esterase$^+$) present in cell mixtures can by highly enriched (to 95% purity) in a single step by rosetting with opsonized erythrocytes followed by sedimentation on Lymphoprep. Separate fractions of highly purified (over 95%) macrophages obtained by the successive use of density gradients and rosetting differ significantly in the production of cytokines, e.g. cells from fractions at lower density produce little IL-6, cells from fractions at higher density are poor producers of TNF-α, while testicular macrophages (TMf) in intermediate fractions produce significant amounts of both cytokines. These differences may suggest that particular subpopulations of testicular macrophages play different biological roles in the testis.

Key words: testicular macrophages; isolation; functional subpopulations; cytokine secretion; FcγR expression.

Introduction

Macrophages are present in the interstitial compartment of the testis in all mammals examined so far, including mice[8], but their role is far from completely elucidated. They are closely associated with Leydig cells by specialized cytoplasmic digitations and can most likely either up- or down-regulate the cellular steroidogenesis of these cells by the release of soluble products[1, 6]. Their interactions with Sertoli cells are by far less clear[2]. There is considerable interest in the role of testicular macrophages (TMf) in the physiology of masculine gonads, but an important technical problem is the separation of macrophages from the majority of other cell types. Several methods have been used up to now, but in general they are laborious and seldom fully successful[4, 6].

To unravel the multiple and intricate functional interactions between TMf and other testicular cells, homogenous cell populations are needed. This requirement is even more crucial as some characteristics regarded as representative for one cell type may be shared by another cell type, such as glass adherence, phagocytosis and IL-6 secretion by both Sertoli cells and TMf[2, 14] or non-specific esterase activity and IL-1 production by Leydig cells and TMf[10].

This paper describes the use of two separation techniques based on different principles to fractionate and

Abbreviations used: TMf – testicular macrophages, MPO – myeloperoxidase.

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purify testicular macrophages. Density gradient centrifugation followed by sedimentation of FcR^+cells by rosetting with opsonized erythrocytes allows one to obtain TMf subfractions of over 95% purity. To further characterize the purified cell populations we used cytological and cytochemical methods as well as *in vitro* production of cytokines.

Materials and Methods

Animals. The eight to 10-week-old male inbred CBA/J mice used were from the breeding unit of the Department of Immunology, College of Medicine, Jagiellonian University, Kraków, Poland.

Reagents. The following reagents were used: Ficoll, Percoll, Lymphoprep (Pharmacia, Uppsala, Sweden); collagenase Type IA, DNase I, Tosyl (Nα-p-tosyl-L-lysine chloromethyl ketone), o-phenylenediamine, hydrogen peroxide, Sigmacote, α-naphtyl acetate, Fast Blue BB salt, glycol monomethyl ether, horse-radish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO); RPMI 1640, fetal calf serum (FCS) (Gibco Life Technologies, Grand Island, NY); trinitrobenzene sulphonic acid (TNBSA) (Eastman Kodak, Rochester, NY); hydroxymethyl aminomethane (Tris) (Serva, Heidelberg, FRG); recombinant mouse IL-6 (PeproTech, Rocky Hill, NY); recombinant murine TNF-α, hamster anti-mouse TNF-α, rabbit polyclonal anti-mouse TNF-α, rat anti-mouse IL-6 (Genzyme, Cambridge, MA); biotinylated rat anti-mouse-IL-6 (Pharmingen, San Diego, CA); and anti-TNP IgG2b mAbs (a gift from Dr. P. A. Flood, University of North Carolina, Chapel Hill, NC).

Isolation of testicular macrophages. TMf were isolated following a slightly modified procedure of HUTSON[6]. The capsule (tunica albuginea) was removed from the testes without cutting the tubules, and the internal (interstitial tissue of the testes) was gently loosened with 30G needles. Then the testes were shaken in Mg^{++} and Ca^{++} free (0.25 and 0.1%, respectively) Dulbecco's phosphate-buffered saline (DPBS) with 0.5% glucose at room temperature for 10 min at 100 cycles/min (non-enzymatic isolation). The remaining interstitial cells were then isolated enzymatically. This part of the isolation was performed using a mixture of 5 ml 0.25% collagenase and 0.1% DNase per 40 testes for 10 min at 37°C in a shaking water bath. Then the enzymatic reaction was stopped with protease inhibitor (Tosyl 6.5 μg/ml) for 5 min. The pool of cells rescued from the interstitial tissue consisted of TMf, Leydig cells and myofibroblasts. Since the efficiency of the non-enzymatic procedure was very low (approximately 2×10^6 cells per testis) both these fractions were collected and these cells were termed thereafter "testicular cells".

The following procedures were used to identify macrophages: 1) glass adherence and phagocytosis; 2) cell surface expression of Fc receptors; 3) cytochemical identification of non-specific esterase and lack of myeloperoxidase (MPO) staining; 4) estimation of monokine secretory activity (TNF-α and IL-6) in the supernatants from the cultures of isolated cells.

Discontinuous density gradient centrifugation. Testicular cells were separated into fractions by sedimentation in Ficoll or by flotation in Percoll gradient[5]. The working solutions of Ficoll were prepared from the stock solution by dilution in DPBS to obtain 15, 14, 13, 12 and 5% concentrations. The solutions of different density (5 ml each) were carefully layered one on top of the other, starting with the heaviest and layering successively lighter layers above. Two $\times 10^7$ cells in 1 ml of DPBS were carefully added on top of the Ficoll gradients in sterile, siliconized 30 ml glass tubes.

Five $\times 10^7$ cells were suspended in 5 ml of 45% Percoll working solution and placed at the bottom of 30 ml glass siliconized, sterile tubes. Then 5 ml each of 39, 33, 27 and 21% solutions of ice-cold Percoll were overlaid. In all gradients the tubes were centrifuged for 30 min at 4°C in a swinging-bucket rotor at 1000 × g for Ficoll, and at 300 × g for Percoll. Cells in all the particular interfaces and the pellet starting at the top of the gradient were collected and washed three times in DPBS to remove residual traces of the gradient medium.

Rosette formation – test for the presence of FcγR I and FcγR II/III. In the direct test for rosette formation, cells in suspension were used. Either mouse (MRBC) or sheep (SRBC) TNP-substituted erythrocytes were employed[11] as indicator cells. To detect the presence of FcγRI/II/III, cells in suspension were incubated for 30 min at 4°C with 5×10^6 TNP-erythrocytes previously opsonized with a subagglutinating dilution of anti-TNP IgG_{2b} mouse mAb (hemagglutination titer 1:640, 45 min at room temp.) and then the number of rosettes was determined microscopically[13].

In all experiments, cells that attached 3 or more TNP-erythrocytes were scored and regarded as FcγR positive and the results were expressed as the percentage of the total cell count. Each experiment was done in duplicate and 100 cells were counted in each group.

In some experiments, 5×10^7 cells in suspension which formed rosettes were layered over 10 ml of Lymphoprep layer, centrifuged at 500 × g (15 min at 4°C), and cells in the pellet were collected.

Histochemistry. Histochemical staining was done in smears of testicular cells fixed in a mixture of acetone and formaldehyde. Non-specific esterase was estimated by the method of KAPLOW[9]. MPO was tested by the method of BRADLEY et al.[3]. Each experiment was done in duplicate and 100 cells were counted in each group. Results were expressed as the percentage of positive cells.

Cytokine immunoassay – measurement of TNF-α and IL-6. Testicular cells were separated by discontinuous density gradients into 5 fractions. Then TMf were obtained by rosetting of separate gradient fractions over Lymphoprep and removing excess erythrocytes by osmotic shock. One $\times 10^5$ viable cells in 0.2 ml RPMI 1640 medium supplemented with 5% FCS were distributed in microwells in flat 96 well Falcon plates and, after 24 h culture, supernatants were collected. Cytokine concentrations in culture supernatants were measured by sandwich ELISA[12] using monoclonal rat anti-mouse IL-6 or monoclonal hamster anti-murine TNF-α antibodies as capture antibodies. As the secondary antibody, biotinylated monoclonal rat anti-mouse IL-6 and HRP-conjugated polyclonal goat anti-rabbit IgG and rabbit anti-mouse TNF-α antibodies were used. The reaction was stopped with 3 M H_2SO_4 and the optical density of each well was measured in a 96-well plate reader at 492 nm. All determinations were done in triplicate. Standard curves were generated by recombinant mouse cytokines. Lower density limits were 30 pg/ml (IL-6) and 10 pg/ml (TNF-α).

Results

We used a modified procedure of HUTSON[6] to recover a mixture of Leydig cells, TMf and myofibroblasts, of which the latter two are glass adherent from interstitial tissue. Using this non-enzymatic procedure, the average cell harvest per testis was only (1.8±0.3) $\times 10^6$ with 4% TMf. When subsequent non-enzymatic and collagenase/DNase treatments were used for isolating cells from interstitial tissue, the average cell harvest was (3.4±1.2) $\times 10^6$ cells with a TMf content of 4–10%. Thus, in further experiments both these fractions were combined.

Of the several one-step procedures we used to isolate the TMf present in the crude mixtures with other testicular cells, only rosetting with opsonized erythrocytes (RBC) followed by sedimentation on Lymphoprep (density 1.077 g/cm^3) or a similar medium (Pancoll, Ficoll-Paque), delivered a highly pure population of macrophages in the pellet (over 95% of TMf). This separation was almost complete, since within the non-sedimenting cells only less then 0.5% were FcγR$^-$ and esterase$^+$ (Table 1). Several other techniques used by us to purify TMf (adherence, phagocytosis, magnetic beads coated with cell surface-directed antibodies) were less efficient and the final contamination of TMf by other cells was in the range of 20–30% (unpublished data). The results of rosetting with TNP-substituted MRBC gave somewhat better yield than TNP-SRBC, moreover, their use prevents the introduction of an additional antigen into the system, so they were preferentially utilized by us in future experiments. Additionally, TMf cell suspensions can be contaminated by monocytes[6] which adhere to the wall of blood vessels or are in transition to enter tissues and can easily be collected using routine procedures. Since these cells are MPO-positive they can be easily discriminated from TMf by histochemical methods. In our experiments MPO-positive cells were either absent in the whole population of testicular cells or within adherent cells or formed only a tiny proportion (below 0.5%) of all FcγR$^+$ cells.

Ficoll discontinuous density gradient sedimentation or, alternatively, Percoll density gradient flotation were used to fractionate testicular cell suspensions. The majority of cells undergoing gradient fractionation accumulated in interface 12/13 of the Ficoll gradient or the

Table 1. Purification of testicular macrophages by sedimentation of rosette-forming cells

TNP-substituted erythrocytes	Direct test (FcγRI, II, III) anti-TNP IgG_{2b} immune complexes (% of cells forming rosettes)	
	non-separated cells	pellet
Sheep	5±2 (< 0.5)*	95±2
Mouse	4±3 (< 0.5)*	96±2

Cells of the male gonads obtained by subsequent non-enzymatic and mild enzymatic treatment were incubated with TNP-derivatized mouse or sheep erythrocytes opsonized previously with anti-TNP IgG_{2b} mAb (immune complexes, direct test). Cells were subjected to sedimentation on Lymphoprep. Number of rosette-forming cells was estimated in the whole non-separated population of cells and in the pellet.

Data represent the mean of 3 independent experiments ± SD.

* Within the non-sedimenting cells after pelleting of FcR$^+$ cells less than 0.5% formed rosettes.

Table 2. Gradient separation of testicular macrophages

Number of fraction	Testicular cells separated on:									
	Ficoll				Percoll				production of	
	range of density (g/cm^3)	interface (%)	cells recovery[a] (%)	rosetting cells[b] (%)	range of density (g/cm^3)	interface (%)	cells recovery[a] (%)	rosetting cells[b] (%)	TNF-α (pg/ml) by 5×10^5 rosetting cells	IL-6 (pg/ml) by 5×10^5 rosetting cells
I	1.030–1.043	9/12	20.4	5	1.030–1.038	21/27	2.3	6	189±5.3	264±141
II	1.043–1.046	12/13	76.2	4	1.038–1.047	27/33	92.0	4	224±3.9	>4000
III	1.046–1.050	13/14	3.1	5	1.047–1.053	33/39	3.2	5	244±8.8	3238±173
IV	1.050–1.055	14/15	0.2	5	1.053–1.059	39/45	1.2	3	41±6.4	963±248
V	>1.055	pellet	0.1	6	>1.059	pellet	1.3	7	36±4.2	558±506

Interstitial cells were subjected to separation on Ficoll sedimentation gradient or Percoll flotation gradient (5×10^7 cells per gradient). Cells from subsequent interfaces and pellets were counted, incubated with opsonized TNP-MRBC and then layered over Lymphoprep and sedimented by centrifugation. In each fraction the percentage of cells forming rosettes with opsonized erythrocytes was estimated. Purified TMf from each fraction were cultured and the levels of TNF-α and IL-6 in supernatants were estimated. All determinations were done in triplicates, and results are expressed as the mean ± SD.

[a] Percentage of recovered cells in each fraction (cells applied to the gradient = 100%).

[b] Refers to the percentage of rosette forming cells in each interface.

27/33 interface of the Percoll gradient (densities 1.043/1.046 and 1.038/1.047 g/cm^3, respectively). The number of cells in other fractions was significantly less (Table 2). Cells from each gradient interface were collected, counted and then the number of rosette-forming cells with opsonized mouse erythrocytes was estimated. The results, summarized in Table 2, from a number of experiments show that macrophages (i.e. rosette-forming cells) were found in all fractions (within the range of buoyant densities (ρ) between 1.030–1.053 g/cm^3), although in different proportions. No one fraction consisted of pure macrophages, and they were contaminated with varying numbers of other cells. Very similar results as regards the presence of FcγR^+ cells in separate fractions were obtained in both types of gradients.

In the following experiment, cell fractions obtained by Percoll flotation were incubated in suspension with opsonized TNP-MRBC and then sedimented on Lymphoprep to obtain highly purified FcγR^+ cells in the pellet. Macrophages free of erythrocytes and dead cells were then cultured *in vitro* for 24 h. The presence of IL-6 and TNF-α in the supernatants was measured by ELISA assay. The results of one such experiment are shown in Table 2. Thus TMf present in gradient fractions I, II and III produced 4–7 times more TNF-α than equal numbers of cells in fractions IV or V. IL-6 was produced in high quantities by TMf in fractions II and III and in moderate amounts by cells in fractions IV and V. Cells in the lightest fraction (I) produced 3 to over 10 times less IL-6 than cells in other fractions. The attachment of immune complexes to macrophages increases significantly their metabolism, release of reactive oxygen intermediates and also production of cytokines. For these reasons our data on IL-6 and TNF-α production by macrophage subpopulations obtained by rosetting techniques may differ from the spontaneous release of these cytokines by unmanipulated cells and, thus, can be used primarily for comparative purposes.

Discussion

Resident testicular macrophages are composed of distinct subpopulations of cells that differ with respect to size, morphology, biochemical and cytochemical characteristics[7], and even by origin[6], since it has been suggested that some TMf of male gonads are not bone-marrow derived but may have a local origin. For these reasons, testing the biological activity of the whole TMf population can be potentially deceptive, since particular fractions may have different or even antagonistic functions.

Our efforts were directed primarily at geting highly purified macrophages from cell mixtures obtained by mechanical and enzymatic treatment of the interstitial tissue of male gonads. In general, the harvest of testicular cells, was rather low (an average 3.4×10^6 cells/testis) and only 4–10% of these cells were TMf: thus, the final output varied between 1.7×10^5 to 3.5×10^5 TMf per testis. A similar percentage of TMf (approximately 12%) among enzymatically isolated testicular cells was found in rats[4].

We have shown that this single-step procedure dependent on the rosetting of testicular macrophages with opsonized mouse erythrocytes and then allowing them to sediment over Lymphoprep, leads to a pure population (over 95%) of cells which by several criteria, are macrophages ($Fc\gamma R^+$, esterase$^+$). The macrophage population obtained by rosetting, however, is not suitable for examining the particular functions of the cells that enter its composition because of their potential functional heterogeneity. Our experiments show that the prior separation of testicular cells on proper gradients combined with the rosetting method delivers five highly purified fractions of TMf differing in buoyant density and demonstrating different potentials to produce IL-6 and TNF-α. The best TNF-α and IL-6 producers were present in intermediate Percoll gradient fractions (27/33, 33/39), while macrophages from fractions at lower density (fraction 21/27) produced little IL-6 and cells from fractions at higher density (39/45 and the pellet) were poor produces of TNF-α. It is, however, necessary to add that since the majority of TMf, accumulated in fraction II (buoyant densities between 1.038 to 1.047), the contribution of cells present in other fractions to the total amounts of lymphokines produced was low. This does not preclude the possibility that these cells play significant and diverse roles in intratesticular regulation. At present it is not clear whether such a significant variability of the spectrum of cytokines released by macrophages present in separate gradient fractions reflects lineage or maturational differences or both. Further characterization of TMf subfractions by such criteria as the expression of cell surface markers, the production of other cytokines, prostaglandins, etc., will allow determining whether they indeed represent subpopulations with different biological functions. Such experiments are currently under way.

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