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Flow Cytometric Analysis of Peripheral T Cell Subsets in the Sialoadenectomized and Salivariadenectomized Male Mice (*Mus musculus* Linn.)

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Abstract

The extraordinary sequential process of T cell development and maturation is hallmark of well-functioning of thymus gland. An earlier study clarifies the relationship between salivary glands and other organs including thymus. In order to define the precise role of salivary gland secreted growth factors on the development, differentiation and maturation of thymocytes, especially CD4 and CD8, we sialoadenectomized (removal of submandibular gland) and salivariadenectomized (removal of submandibular and sublingual glands) the male albino mice. The mice were operated at the age of 20 days and maintained under normal conditions in the animal house along with control, up to the age of ten weeks. Subsequently, blood samples were collected and peripheral T cell subsets was analysed using FACSCalibur flow cytometer with BD Tritest CD4FITC/CD8PE/CD3 PerCP reagent. It was observed that in the absence of salivary gland-secreted growth factors, especially EGF, the mature naïve T cells output gets disturbed, and there was significant reduction in CD4 absolute and % count and CD4:CD8 ratio, signifying the importance of salivary gland-secreted growth factors in maturation of immune cells in the thymus. It is suggested that the importance of interplay of hormones and neuropeptides on one hand and salivary secretory regulatory peptides on the other, on the T cell differentiation and maturation in the thymus is investigated.

Keywords: CD4 and CD8 Thymocytes, Flow Cytometry, Salivariadenectomy, Sialoadenectomy

1. Introduction

The well-co-ordinated and orderly process of T cell expansion and maturation is co-ordinated by the thymus gland. The thymus gland has been believed to be a mysterious organ from ancient times[1]. However, in the more recent times knowledge about this organ has been greatly expanded in view of extensive research; particularly, it has been proved that this organ plays unique role in development and maturation of T lymphocytes that are the key element that co-ordinate immune responses and thus play critical role in cell-mediated immunity[2].

The developmental process of naïve T cells begins in the bone marrow where common lymphoid progenitor cells develop. Then the cells migrate to the thymus and eventually commit to specific T-cell lineages. Based on the co-expression of CD4 and CD8, three major stages of thymic αβ T-cell development have been defined: DN (double negative) stage ($CD4^-$/low$CD8^-$); DP (double positive) stage ($CD4^+CD8^+$); and SP (single positive) stage ($CD4^+CD8^-$ or $CD4^-CD8^+$)[3]. Apart from being the main source of all T cells, thymus is the region where T cells diversify and then are shaped into an effective primary T-cell repertoire by an extraordinary pair of selection processes[4]. During the process of positive and negative selection, αβ thymocytes also commit to either the $CD4^+$ (normally MHC class II restricted) or $CD8^+$ (normally MHC class I restricted) T cells[3].

Upon maturation $CD4^+$ and $CD8^+$ T cells leave the thymus and enter the peripheral circulation. Naive T

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cells continually recirculate between the blood and lymphatic systems. If a naive cell recognizes an antigen-MHC complex on an appropriate antigen-presenting cell or target cell, it will be activated, initiating a primary response. T cells activated in this way divide 2 to 3 time per day for 4 to 5 % days, generating a clone of progeny cells, which differentiate into memory or effector T-cell population[4]. Two clonally expanded populations originate from this primary response: 'effector' cells, which combat spread of the pathogen; and 'memory' cells, which guard against subsequent infections. So, this stepwise development and maturation process is most important to defend our body from all the types of foreign agents. The healthy population of T cell subsets in the peripheral blood is a reflection of well-functioning of the thymus gland.

The salivary gland is considered to be a reservoir of many growth factors in rodents. The sub-mandibular gland (SMG) secretes a number of growth factors including epidermal growth factor (EGF), nerve growth factor (NGF), mesodermal growth factor (MGF), transforming growth factor-alpha (TGFα) etc[5]. Studies on the effect of growth factors secreted by the sub-mandibular gland on various organs are being carried out in different laboratories. Numerous factors of different origin are involved in regulation and modulation of morphogenesis and homeostasis of the T-cell system. Several studies were carried out which indicated a marked effect of sub-mandibular gland on T- cell system. Depletion in thymocytes causes atrophy of thymus gland[6]. Reduction in body weight and thymus gland weight after sialoadenectomy has been shown in rat[6-10]. We found in our previous studies that in the absence of growth factors secreted by sub-mandibular gland there was decrease in the weight of thymus gland[11]. Subsequent histological and histochemical studies revealed that reduction in the size of medulla indicating reduction in the number of thymocytes and decrease in the Hassall's corpuscles with many signs of early atrophy of thymus gland[12]. We have also found that after sialoadenectomy and salivariadenectomy there was decrease in the rate of T-cell maturation with reduction in intensity of immunohistochemical staining and decrease of CD3 and CD5 cells[13]. In this background we undertook to find the effect of sialoadenectomy and salivariadenectomy on T cell maturation and peripheral T cell subset count for which we adopted flow cytometric analysis of blood sample.

2. Material and Methods

Ten weeks old male Swiss albino mice (*Mus musculus*) were used in the experiments. The animals were housed in departmental animal house (1852/PO/EReBi/S/15/CPCSEA) under approval of Institutional Animal Ethical Committee (Approval reference-03/2012) and proper housing conditions. They were provided with food (Amrut mice feed) and water *ad libitum*. For the present study 18 numbers of twenty day old mice weighing 8 to 15 g were randomly divided into three groups: sham operated sialoadenectomized and salivariadenectomized. Bilateral sialoadenectomy (removal of submandibular gland), bilateral salivariadenectomy (removal of sub-mandibular and sub-lingual glands) and sham operation were carried out. Sham-operated animals were subjected to procedures identical to -ectomy except that glands were not removed.

2.1 Collection of Blood Samples

After completion of 10 weeks since the surgery, blood sample was collected. It was done aseptically by vein puncture at tail region, and stored in sterile K3 EDTA BD Vacutainer blood collection tubes (Lavender top) and mixed well. The anticoagulated blood samples were stored at room temperature (20-25^{0}C).

2.2 Flow Cytometry

The lymphocyte subsets were analysed on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, BD FACSCalibur system, with automated sample loader and computer hardware with BD CellQuest Pro™ software). Here BD Tritest CD4FITC/CD8PE/CD3 PerCP reagent was used with BD Trocount™ absolute count tubes for identification and determination of absolute counts of mature T lymphocytes ($CD3^+$), helper/inducer ($CD3^+CD4^+$) T lymphocytes and suppressor/cytotoxic ($CD3^+CD8^+$) T lymphocytes in 1 µl of erythrocyte-lyzed whole blood as per manufacturer's instructions. The FACSCalibur was calibrated and reconfirmed daily using the BD Calibrite beads and BD-FACSComp software (version 2.0). A control sample of commercially available whole blood was run daily to optimize the instrument settings. Preceding the flow cytometry the absolute and percent white blood cell (WBC) count was obtained. For the present analysis 50 µl anticoagulated whole blood samples were mixed with BD Tritest reagent using

Table 1. Effect of sialoadenectomy and salivariadenectomy on peripheral CD3, CD4 and CD8 cells in the thymus gland of male mice studied by flow cytometry

Sr. No.	Parameter	Control (n=6)*	Sialoadenectomy (n=6)*	Salivariadenectomy (n=6)*
1.	Total Lymph (%) W.B.C.s	75.44 ±1.1675	41.28 ± 1.359	40.32 ± 1.2701
	Statistical significance	1:2, P < 0.01	2:3, P < 0.01	1:3, P >0.5
2.	CD3 %	26.662 ± 0.8506	15.922 ± 0.3638	6.632 ± 0.2648
	Statistical significance	1:2, P < 0.01	2:3, P < 0.01	1:3, P < 0.01
3.	CD4%	20.396 ± 1.0242	3.83 ± 1.138	1.526 ± 0.391
	Statistical significance	1:2, P < 0.01	2:3, P < 0.01	1:3, P < 0.05
4.	CD8 %	4.472 ± 0.7337	10.8 ± 1.3637	3.532 ± 0.2663
	Statistical significance	1:2, P < 0.01	2:3, P < 0.01	1:3, P < 0.01
5.	CD4:CD8 ratio	6.1± 1.581	0.288 ± 0.0683	0.266 ± 0.0288
	Statistical significance	1:2, P < 0.01	2:3, P < 0.01	1:3, P > 0.5

- Data are expressed as Mean ± SD (P < 0.05= almost significant, P < 0.01= significant, P < 0.001= highly significant, P > 0.5-non significant)
- Number in parenthesis denotes the number of animals.

vortex mixer, and incubated for 15 minutes in the dark at room temperature (20-25^{0}C).Then 2 ml of BD FACS lysing solution was added and centrifuged at 3000 rpm (7 to 8 minutes). Again samples were centrifuged twice by adding 2 ml for first then 500 ml of sheath fluid. This sample (working solution) was then analysed with the help of FACSCalibur flow cytometer. The acquisition and analysis of data were carried out with the help of BD Cell Quest Pro software. The observations were recorded.

2.3 Statistical Treatment of Data

The acquisition and analysis of data were carried out with the help of BD Cell Quest Pro software. The statistical analysis was performed using one way Analysis of variance (ANOVA) followed by Tukey's Post Hoc Test. All values were expressed as mean ± S.D. (P < 0.05= almost significant, P < 0.01= significant, P < 0.001= highly significant, P > 0.5-Non Significant).

3. Results

The table shows data from flow cytometric analysis of peripheral CD3, CD4 and CD8 T cell subsets in sialoadenectomized and salivariadenectomized male mice. The % counts of WBC's, CD3 and CD4 were decreased significantly in sialoadenectomized group and further decreased in salivariadenectomized group as compared to control. The decrease in salivariadenectomized group was more significant than in sialoadenectomy group. But the % count of CD8 was increased in sialoadenectomized group as compared to salivariadenectomized group. The CD4:CD8 ratio decreased significantly in sialoadenectomized group but the reduction in CD4:CD8 ratio in salivariadenectomized group was not significant as compared to control.

4. Discussion

The absolute counts and percentage of T-lymphocyte subsets in peripheral blood provide evidence about the status of development and well-functioning of the immune defence system[14]. The maturation of T cells in the thymus is a highly organized process. Any deviation can lead to immunodeficiency, autoimmunity, or cancer. Flow cytometry is the golden standard for the estimation of T cell population[15].

The major T cell subset counts i.e., helper/inducer lymphocytes are a subset of T lymphocytes ($CD3^+$) that are $CD4^+$ and suppressor/cytotoxic lymphocytes are a subset of T lymphocytes ($CD3^+$) that are $CD8^+$ are used to characterize and monitor some forms of immunodeficiency[16-18] and autoimmune diseases[19,20]. Individuals with HIV typically exhibit a steady decrease of helper/inducer CD4 T lymphocyte counts as the infection progresses[21]. Suppressor/cytotoxic lymphocyte, i.e. CD8, cell counts lie outside the normal reference range in some

autoimmune diseases[22] and in certain immune reactions such as acute graft-versus-host disease (GVHD)[23]. The $CD8^+$ subset count is elevated in many patients with either congenital or acquired immune deficiencies, such as severe combined immunodeficiency (SCID)[16] or acquired immune deficiency syndrome (AIDS)[24].

According to a few studies the $CD4^+$ T cell count has been shown to be influenced by sex, age, race, time of specimen collection (diurnal rhythms), physical and psychological stress, pregnancy, drug administration (zidovudine, cephalosporin, cancer chemotherapeutics, nicotine and steroids), tuberculosis, viral infections, presence of anti-lymphocyte auto-antibodies and procedures like splenectomy[25,26].

Epidermal Growth Factor (EGF) secreted by the salivary gland brings about modification in immune responsiveness. This protein, present in high concentrations in mouse submandibular glands, is capable of modifying development [27-29]. Specific receptors of EGF have been detected in a variety of tissues, including the membranes derived from the mouse thymus[30-32]. But its actual role in thymic physiology is still unclear. It has been reported that exogenous EGF promotes a dose-dependent modulation of thymocyte development in foetal thymus organ cultures (FTOC). Insoluble form of exogenous EGF blocked thymocyte growth and differentiation which acts at double negative to double positive transition. Such blockade alters TCR $\alpha\beta^+$ thymocyte subset, so they were absent and TCR $\gamma\delta^+$ subsets were found among EGF FTOC culture[33]. Removal of salivary glands has been shown to abolish salivary EGF and cause decreased plasma EGF level. Therefore, in absence of salivary EGF there may be disruption in cell growth, proliferation and differentiation of T lymphocytes in the thymus in the sialoadenectomized and salivariadenectomized mice. It may be possible that due to disturbance in differentiation in the absence of EGF after sialoadenectomy and salivariadenectomy, the mature naïve T cells output may get disturbed causing reduction in CD4 absolute and % count and CD4:CD8 ratio.

It is of interest to note that EGF, especially of salivary gland origin, is regulated by hormones[28,29]. Further, the role of thymus in T cell differentiation and maturation is under modulation by hormones and neuropeptides that are complex and multifaceted[10,34,35]. Therefore, it would be pertinent to investigate the interplay of thymic regulatory hormones and salivary gland regulatory peptides.

5. Conclusion

From the present investigation it is clear that in the absence of salivary gland-secreted growth factors, especially EGF, the mature naïve T cells output get disturbed and there would be reduction in CD4 absolute and % count and CD4:CD8 ratio. Investigation on the importance of the interplay of hormones and neuropeptides on one hand and salivary secretory regulatory peptides on the other on the T cell differentiation and maturation in the thymus is pertinent.

6. References

1. Schmeck HM Jr. Mysterious thymus gland may hold the key to aging. New York Times. 1982: C:1. https://www.nytimes.com/1982/01/26/science/mysterious-thymus-gland-may-hold-the-key-to-aging.html
2. Bosire EM, Nyamache AK, Gicheru MM, et al. Population specific reference ranges of CD3, CD4 and CD8 lymphocyte subsets among healthy Kenyans. AIDS Res Ther. 2013; 10(1):24. doi: 10.1186/1742-6405-10-24.
3. Wang Y, Edinger M, Mittar D, Mclntyre C. Studying mouse thymocyte development using multiparametric flow cytometry: An efficient method to improve an 8-colorp on the BD FACSVerse™ System. BD Biosciences, San Jose, 1. 2012.
4. Kindt TJ, Goldsby RA, Osborne BA. Kuby Immunology. 6th Edition, Revised. W.H. Freeman Publication and Co. Ltd. New York. U.S.A. 2006.
5. Barka T. Biologically active polypeptides in submandibular glands. Histochem Cytochem. 1980; 28:836-59.
6. Martinez-Hernandez A, Nakane PK, Pierce GB. Relationship between submaxillary gland and the thymus. Lab Invest. 1973; 29(2): 266-71.
7. Letic-Gavrilovic A, Jankovic BD. Effects of submandibular salivary gland on lymphoid tssue in rats. Periodicum Biologorum. 1983; 85(3):103-4.
8. Lectic-Gavrilovic A, Jankovic BD. Immunological reactions in rats treated with different extracts of submandibular salivary gland. Periodicum Biologorum (Supp-1). 1986; 88:154-55.
9. De Lange GL. The effect of salivary gland extracts on the histology of lymphoid organs and salivary glands in mice. Arch Oral Biol. 1975; 20:515-20.
10. Dean DH, Hiramoto RN. Effects of submandibularectomy and castration on thymus and spleen weights in male mice. Experientia. 1983; 39(12):1351-2.
11. Walvekar MV, Khairmode SP, Potphode NT. Alkaline phosphatase activity and DNA content in thymus gland of sialoadenectomised male mice. DAMA Int. 2012; 1(2):5-7.

12. Walvekar MV, Khairmode SP, Desai SR. Effect of salivary gland secreted growth factors on histology of thymus gland in mice. Int J Curr Biotechnol. 2014; 2(4):1-9.
13. Walvekar MV, Desai SR, Khairmode SP, Pillai MM. Role of salivary glands secreted growth factors on differentiation of CD3 and CD5 cells of thymus in mice. Asian J Microbiol Biotech Env Sc. 2016; 18(1):249-56.
14. Westermann J, Pabst R. Distribution of lymphocyte subsets and natural killer cells in the human body. Clin Investig. 1992; 70(7):539-44.
15. Shete A, Thakar M, Abraham PR, Paranjape R. A review on peripheral blood CD4+ T lymphocyte count in healthy adult Indians. Indian J Med Res. 2010; 132(6):667-75.
16. Schmidt RE. Monoclonal antibodies for diagnosis of immunodeficiencies. Blut. 1989; 59(3):200-6.
17. Nicholson JKA. Use of flow cytometry in the evaluation and diagnosis of primary and secondary immunodeficiency diseases. Arch Pathol Lab Med. 1989; 113(6):598-605.
18. Foucar K, Goeken JA. Clinical application of immunologic techniques to the diagnosis of lymphoproliferative and immunodeficiency disorders. Lab Med. 1982; 13(7):403-13.
19. Cohen SB, Weetman AP. Activated interstitial and intraepithelial thyroid lymphocytes in autoimmune thyroid disease. Acta Endocrinol. (Copenh) 1988; 119(2):161-6.
20. Smolen JS, Chused TM, Leiserson WM, et al. Heterogeneity of immunoregulatory T-cell subsets in systemic lupus erythematosus: correlation with clinical features. Am J Med. 1982; 72(5):783-90.
21. Landay A, Ohlsson-Wilhelm B, Giorgi JV. Application of flow cytometry to the study of HIV infection. AIDS. 1990; 4(6):479-97.
22. Antel J, Bania M, Noronha A, Neely S. Defective suppressor cell function mediated by T8+ cell lines from patients with progressive multiple sclerosis. J Immunol. 1986; 137(11):3436-9.
23. Gratama JW, Naipal A, Oljans P, et al. T-lymphocyte repopulation and differentiation after bone marrow transplantation: early shifts in the ratio between T4+ and T8+ T lymphocytes correlate with the occurrence of acute graft-versus-host disease. Blood. 1984; 63(6):1416-23.
24. Giorgi JV, Hultin LE. Lymphocyte subset alterations and immunophenotyping by flow cytometry in HIV disease. Clin Immunol Newslett. 1990; 10(4):55-61.
25. Maini MK, Gilson RJ, Chavda N, et al. Reference ranges and sources of variability of CD4 counts in HIV-seronegative women and men. Genitourin Med. 1996; 72(1):27-31.
26. Miyawaki T, Taga K, Nagaoki T, et al. Circadian changes of T lymphocyte subsets in human peripheral blood. Clin Exp Immunol. 1984; 55(3):618-22.
27. Cohen S. Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. J Biol Chem. 1962; 237:1555-62.
28. Byyny RL, Orth DN, Cohen S, Doyne ES. Epidermal growth factor: effects of androgens and adrenergic agents. Endocrinology. 1974; 95(3):776-82.
29. Roberts ML. Testosterone-induced accumulation of epidermal growth factor in the submandibular salivary glands of mice, assessed by radioimmunoassay. Biochem Pharmacol. 1974; 23(23):3305-8.
30. O'Keefe E, Hollenberg MD, Cuatrecasas P. Epidermal growth factor: characteristics of specific binding in membranes from liver, placenta and other target tissues. Arch Biochem Biophys. 1974; 164(2):518-26.
31. Lazorick Le PTS, Whichard LP, Haynes BF, Singer KH. Regulation of cytokine production in the human thymus: epidermal growth factor and transforming growth factor α regulate mRNA levels of interleukin 1 α (IL-1α), IL-1 β, and IL-6 in human thymic epithelial cells at a post transcriptional level. J Exp Med. 1991; 174(5):1147-57.
32. Screpanti I, Scarpa S, Meco D, et al. Epidermal growth factor promotes a neural phenotype in thymic epithelial cells and enhances neuropoietic cytokine expression. J Cell Biol. 1995; 130(1):183-92.
33. Freitas CS, Sergio R, Dalmaua'b KK, Wilson S. Epidermal growth factor (EGF) modulates fetal thymocyte growth and differentiation: partial reversal by Insulin, mimicking by specific inhibitors of EGF receptor tyrosine kinase activity and differential expression of CD45 phosphatase isotypes. J Immunol. 1998; 161(7):3384-92.
34. Savino W, Dardenne M. Endocrine control of thymus physiology. Endocr Rev. 2000; 21(4):412–43.
35. Savino W, Mendes-da-Cruz DA, Lepletier A, Dardenne M. Hormonal control of T-cell development in health and disease. Nat Rev Endocrinol. 2016; 12(2):77-89.