



دراسة مناعية للمرضى المصابين بالتهاب اللوزتين البكتيري المتكرر

أطروحة مقدمة من قبل

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وهي جزء من متطلبات نيل درجة دكتوراه فلسفة في

علوم الحياة - الأحياء المجهرية

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رمضان - 1427

أيلول - 2007

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

﴿ وَعِنْدَهُ مَفَاتِحُ الْغَيْبِ لَا يَعْلَمُهَا إِلَّا هُوَ وَيَعْلَمُ مَا فِي الْبَرِّ وَالْبَحْرِ
وَمَا تَسْقُطُ مِنْ وَرَقَةٍ إِلَّا يَعْلَمُهَا وَلَا حَبَّةٍ فِي ظُلْمَتِ الْأَرْضِ وَلَا
رَطْبٍ وَلَا يَابِسٍ إِلَّا فِي كِتَابٍ مُبِينٍ ﴾

صَدَقَ اللهُ الْعَظِيمُ

من سورة الانعام (59)

الخلاصة

هدفت الدراسة الحالية الى مقارنة المناعة الخلوية والخلطية التي تحدث على مستوى المناعة الجهازية والمناعة الموضعية للمرضى المصابين بالتهاب اللوزتين المتكرر والناتج عن البكتيريا الموجبة لصبغة غرام خلال الفترة 2006-2007 . شملت الدراسة 103 مريضا راجعوا مستشفى رزكاري التعليمي في أربيل لغرض أستئصال اللوزتين و 40 شخصا بنفس الاعمار أعتبروا كمجموعة سيطرة . لكل مريض تم أخذ عينة دم (مع مانع تجلط وبدون مانع تجلط) وكذلك اللوزتين.

تم مسح الاحياء المجهرية المرتبطة بالجزء الحشوي للوزتين وتم التركيز على البكتيريا الموجبة لصبغة غرام متضمنة البكتيريا العنقودية *S.aureus* و البكتريا المسبحية *S.pyogenes* وخليط من هذه البكتيريا.

و درست كفاءة خلايا متعددة اشكال النوى بأستعمال طريقة أختزال صبغة النايترولوتيترازوليوم وكانت فعالية أختزال الصبغة طبيعيا بين المرضى.

وتم دراسة المناعة الخلوية من خلال دراسة عامل تثبيط هجرة الخلايا اللمفاوية وقابلية الخلايا اللمفاوية لتكوين التشكيل الزهري ولقد لوحظت مظاهر التحمل الناتج عن وجود المستضدات البكتيرية بالنسيج مع استجابة جزئية في المناعة الجهازية.

كما وجد ان عيارات الاجسام المضادة المتخصصة في المناعة الموضعية (اللوزتين) أعلى نسبيا مقارنة بالمناعة الجهازية التي اظهرت نسبة طبيعية مقارنة بالاصحاء.

أما تركيز الساييتوكاينات (الانترليوكين-6 والانترفيرون-كاما) كانت طبيعيه في المناعة الجهازية مع ارتفاع معنوي ($P<0.001$) للانترليوكين-10 مقارنة بالاسوياء , بينما تركيز الساييتوكانات (الانترليوكين-6 والانترليوكين-10 والانترفيرون-كاما) في المناعة الموضعية كان مرتفعا معنويا وهذا يعكس التحفيز المستمر بواسطه المستضدات الموجودة في التجويف البلعومي الفمي.

كما وجد ان البكتريا المسبحية *S.pyogenes* تكون اكثر فعالية في تحفيز الانترليوكين-10 والانترفيرون-كاما واكثر تثبيطا لقابلية التشكيل الزهري في المناعة الجهازية وتظهر تاثيرات مختلفه على الجهاز الموضعي بينما بكتريا العنقودية *S.aureus* اكثر كفاءة في تحفيز الاجسام المضادة المتخصصة في اللوزتين .

وهذه النتائج اثبتت ان التهاب اللوزتين المتكرر يحفز المناعة الموضعية ويظهر تاثيرا أقل على استجابته المناعة الجهازية من هذا يمكن التخمين بأن المناعة الموضعية في اللوزتين تعمل بصورة منفصلة عن

المناعة الجهازية. كما وجد ان الاعداد بين 5-30 سنة لا يظهر فرقاً معنوياً في التأثير على قابلية البلعمة للخلايا المتعددة الاشكال النووي، ولكن اختبار عامل تثبيط هجرة الخلايا للمفاويه أظهر بأن الاطفال بين 5-9 سنوات اكثر حساسية للمستضدات البكتيرية من البالغين (20-30 سنة) بينما نتائج المرضى بسن البلوغ والمراهقة اظهرت استجابات مناعية متباينة.

وتم دراسته خمسة عشرة لوزة حنكية بواسطة المجهر الضوئي وتركز الاهتمام على الطبقة السطحية الظهارية والاخايد بالاعتماد الى ما يعزى اليه من وظائف مناعية. وهذه الطبقة غير متناسقة تحوي مواضع تكون فيها الطبقة الظهارية حرشفيه غير متقرنه ومواضع شبكية تشبه الاسفنج ودرجه التشابك تختلف حسب نوع البكتيريا التي تاويها اللوزتين .

وقد لوحظ ان البكتريا المسبحية *S.pyogenes* تسبب نوع من التهيج بالطبقة الظهارية مسببتا زياده في سمك تلك الطبقة.

كما وانه لم يشاهد هجرة الخلايا المتعددة اشكال النوى أو تواجدها في الافرازات السطحية في اللوز التي تاوي البكتريا المسبحية *S.pyogenes* وتفسير ذلك يعود الى عامل الضراوه (C5a peptidase) الذي يحطم عوامل الجذب الكيميائي التي تتحفز عن طريق تحفيز جهاز المتمم البديل وبالتالي تثبيط تجنيد خلايا متعددة اشكال النوى للهجرة الى مكان الاصابه.

أما النسيج الحشوي للوزتين خارج الحويصلات (Extra follicular region) فقد اظهر زيادة معنوية ($P<0.01$) في خلايا البلازما للوز التي تاويها البكتريا العنقودية *S.aureus* .

وقد لوحظ على اللوز التي تاويها البكتريا المسبحية *S.pyogenes* ضمور الى تضخم قليل مع عدد قليل من الجريبات ذات الحدود غير الواضحه مع تنخر وتحطم نسيج اللوزتين.بينما اللوز التي تاويها البكتريا العنقودية *S.aureus* غالبا ما تكون متضخمه مع زياده معنويه ($P<0.001$) في عدد الجريبات التي تتميز بحدود واضحة .



Immunological Study of Patients with Recurrent Bacterial Tonsillitis

A Thesis Submitted By

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To the Council of College of Science University of Babylon

In Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy in

Biology – Microbiology

Supervised by

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Dedication

To My Mother

Certification

We certify that this thesis was prepared under our supervision at the Department of Biology, College of Science, University of Babylon as a partial of fulfillment for the requirements of the degree of Doctor of Philosophy in Biology (Microbiology) and this work has never been published anywhere.

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Acknowledgements

I would like to express my sincere thanks to Prof., Dr. Ibrahim M.S. Shnawa and Assist. Prof., Dr. Farhad J. Khayyat for their guidance, encouragement, wisdom, and patience throughout this work.

Thanks to the University of Babylon, College of Science and Biology department for providing me with the opportunity to complete my Ph.D. studies and for their continuous help. Thanks to the University of Salahaddin, College of Science and Biology Department for their continuous help. Special thanks to Rizgary Teaching Hospital in Erbil city for providing me with the research environment to carry out this work in their bacteriological and histological laboratories.

I would like to express my sincere thanks to the following people for their invaluable assistance:

*Dr. Salah A.B. Ali, Miss Haifa A. Baker, and Miss Aveen A. Hawez.

For their assistance and help with tissue processing and analysis of histological sections.

*Dr. Frial G. Abd and Miss. Demen Aula

For their generous guidance in the field of practical works.

*Dr. Elham B. Shabo, Dr. Didar Hayder Ahmad, and Miss. Namam R. Abbas.

In Directorate of Central Veterinary Laboratory in Erbil for their help to carry out ELISA work in their ELISA department.

*Dr. Ismael M. Maulood for his generous guidance and assistance in the field of statistical analysis.

Taban Kamal Rasheed

Abstract

The present study was aimed to comparing cellular and humoral immune response occurring at tonsillar mucosa and systemic level in recurrent tonsillitis patient admitted for tonsillectomy (RT-TE) harbors gram positive bacteria in their tonsils. This work was carried out between 2006-2007. The study comprised one hundred and three patients who attended to Rizgary Teaching Hospital in Erbil city for tonsillectomy as well as forty age match controls. For each of the patients and control groups' blood with and without anticoagulants as well as tonsillar tissue from patient were collected.

The microbial profile associated with the core of the tonsils was determined with focusing on gram positive bacteria including *Staphylococcus aureus*, *Streptococcus pyogenes*, and mixed bacteria.

Phagocytosis using nitroblue tetrazolium (NBT) dye reduction was evaluated in peripheral blood, normal NBT dye reduction was observed among RT-patient.

Both LIF and E-rosette forming cells show tolerance phenomenon and antigen induced suppression in the tonsillar tissue with mild response in systemic immunity. Serum specific antibody was higher in tonsillar tissue than systemic which show a low increase to normal range.

Normal concentration of interleukine-6 (IL-6) and interferon- γ (IFN- γ) was observed in systemic immune response with significant increase ($P < 0.001$) in IL-10 concentration in comparison with control group, while mucosal IL-6, IL-10, and IFN- γ concentration was highly increased reflecting constant stimulation by environmental antigen encountered in the oropharyngeal cavity.

S.pyogenes in the RT-patient more effective in inducing IL-10 and IFN- γ , inhibit systemic E-rosette and show different effects on mucosal tissue, while *S.aureus* are more effective in inducing specific Ig in the tonsils.

These results suggest that RT associated with local immunity and show lesser effect on systemic immune response speculating that tonsillar immune system function independently from systemic immune apparatus.

Age between (5-30yr.) had no significant effect on phagocytic activity of polymorph nuclear neutrophils (PMN). However, leukocyte migration inhibition factor (LIF) test indicated that children (5-9yr.) are more sensitive to bacterial Ag than adult (20-30yr.). While, puberty and adolescent group shows different immunological responses.

Fifteen human palatine tonsils were studied by light microscope. Special attention was given to the epithelium and the crypts in the context of its ascribed immunological function. This epithelium was not uniform and contained patches of stratified squamous non keratinizing epithelium and patches of reticulated sponge-like epithelium. The degree of reticulation in epithelial cell was varied according to different bacteria harbors in tonsils. *S.pyogenes* had irritative effect on the epithelium causing increase in thickness.

Neither presence, nor migration of polymorph nuclear neutrophils (PMN) to the surface secretion was observed in RT patients harbors *S.pyogenes* in their tonsils. This explained through streptococcal virulence factor C5a peptidase which destroyed the chemotactic signal generated by the alternative complement pathway, there by inhibiting recruitment of PMN to the site of infection.

The numerical density of plasma cell increased significantly ($P<0.01$) in the extrafollicular area of those harbors *S.aureus*.

Atrophied to mild hypertrophied tonsils with less number of follicles which have ill-defined borders association with damage and necrosis was observed in tonsils harbors *S.pyogenes*, while tonsils harbors *S.aureus* frequently hypertrophied with significantly ($P<0.001$) higher number of follicles which have defined borders.

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Abbreviations

Ab	Antibody
Ag	Antigen
APC	Antigen presenting cell
ASL	Air way surface liquid
CfC	Cell free culture
CB	Centroblast
CC	Centrocyte
DCs	Dendritic cells
E-RFC	E-rosette forming cell
FDC	Follicular dendritic cell
GC	Germinal center
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-monocyte colony-stimulating factor
GAS	Group A Streptococci
HEV	High endothelial venule
HBD	Human B-defense
IgA	Immunoglobulin-A
IgG	Immunoglobulin-G
IgM	Immunoglobulin-M
IDCs	Interdigitating dendritic cells
IFN- γ	Interferon- γ
IL-1	Interlukin-1
IL-2	Interlukin-2
IL-4	Interlukin-4
IL-6	Interlukin-6
IL-8	Interlukin-8
IL-10	Interlukin-10
LIF	Leukocyte migration inhibition factor
LPS	Lipopolysaccharid
LuK-Pv	Panton-Valentine leukocidin
MALT	Mucosal-associated lymphoid tissue
NBT	Nitroblue tetrazolium
PMN	Polymorph nuclear neutrophils
PPP	Pustulosis palmaris et plantaris
RT-TE	Recurrent tonsillitis patient admitted for tonsillectomy.
sRBC	Sheep red blood cell
SCP	Streptococcal C5a peptidase
TLRs	Toll-like receptors

Tig	Tonsillar immunoglobulin
TTL	Tonsillar tissue lymphocyte
TGF- β	Transforming growth factor- β
TNF- α	Tumor necrosis factor- α

Introduction

Palatine tonsils are secondary lymphoid organs located at a critical position for immunological detection of airborne and ingested antigens (Claeys *et al.*, 2003). They are sites where innate immunity such as physical and chemical barriers, phagocytic cells and humoral factors as complement and cytokine leads to onset of the adaptive immunity mediated by B and T lymphocytes (Von Andrian and Mempel, 2003).

Nave lymphocytes circulated between the blood and secondary lymphoid organs. Once antigens are encountered with a secondary lymphoid, the passing nave B and T lymphocytes bearing specific antigen receptors are retained. After being activated, they undergo growth, proliferation, clonal expansion, acquired effectors function and immunological memory (Mansson *et al.*, 2006).

Xie *et al.*, (2004) reveals that immunoglobulin-G (IgG) containing B cell predominate in all tonsil compartments, including follicles, extra follicular area and reticular epithelium, whereas immunoglobulin-A (IgA) cells are found predominantly in extra follicular areas, especially sub epithelial area and immunoglobulin-M (IgM) cells are in follicles.

The immune cells of the tonsils tissue contain predominantly B cells (approximately 65 %); approximately 30 % CD3+ T cells, and 5% macrophages. 80 % of the T cells were primarily of the helper subset (Boyaka *et al.*, 2000).

Andersson *et al.*, (1994) indicated that cytokine which involved in the regulation of immune processes are predominantly produced at sites of local antigen stimulation and produced by intraepithelial lymphocyte, lymphoid cells, and non-lymphoid cells including interleukin-2(IL-2), interleukin-4(IL-4), interleukin-6(IL-6), tumor necrosis factor- α (TNF- α), lymphotoxin- α (LT- α), interferon- γ (IFN- γ) and transforming growth factor- β (TGF- β), while a few cytokines interleukin-8(IL-8) and interleukin-1(IL-1) can be found in the crypt epithelium with total absence of cytokine in the extra follicular region.

Tonsillitis occurs when trapped organisms multiply within and on the tonsil when virulence and activity of pathogenic organisms in the tonsillar lymphoid tissue exceed the protective potency of activated lymphoid and immunoglobulin producing cells. Numerous researches indicate the polymicrobial nature of the deep tonsillar flora in patient with chronic tonsillitis (Brook *et al.*, 1981; Brook and Yocum, 1984). *Staphylococcus aureus* and beta-hemolytic streptococci are among the predominant isolates obtained from patient suffering from recurrent tonsillitis (Abhay *et al.*, 2005).

Histologically, chronically inflamed tonsils show epithelial changes with an initial increase in reticulated epithelium to cover the inter follicular areas as well as the follicles (Scadding, 1990). Beneath the epithelium the lymphoid tissue had similar histological properties of normal human tonsils, the only detectable difference being the presence of more and larger follicles in the hypertrophied tonsils (Ozbilgin *et al.*, 2004).

The purpose of this work was to determine:

- 1- Bacterial profile in the core of the tonsils.
- 2- The nature of cellular and humoral immune response occurring at the mucosal and systemic level.
- 3- The similarities and differences in immune response among recurrent tonsillitis patient admitted for tonsillectomy (RT-TE) harbors different bacteria including *S.aureus*, *S.pyogenes* or mixed bacteria in their tonsils.
- 4- Mucosal and systemic cytokine expression during recurrent tonsillitis including IL-6, IL-10, and IFN- γ .
- 5- Histological modification or damage at mucosal level induced by immune responses or mucosal pathogen.
- 6- The effect of age on systemic and mucosal immune response in RT-TE harbors different bacteria including *S.aureus*, *S.pyogenes* or mixed bacteria in their tonsils.

2- Review of Literatures

2.1: The palatine tonsils:

The paired palatine tonsils are located at the transition of the mouth to the oropharynx, the common entry of both the gastrointestinal and the respiratory tract. This significant position implies a key role of the palatine tonsils as secondary lymphoid organs in initiating immune responses against various antigens that enter the body through the mouth and the nose (Brandtzaeg, 1984).

The palatine tonsil is a lymphoepithelial organ and belongs to the integrated mucosal immune system of the pharynx (Ogra et al., 2001). They are frequently affected by bacterial and viral infection causing local inflammation and systemic reaction (Kasenomm *et al.*, 2005).

Waldeyer describes a ring of lymphoid tissues in the human pharynx. Beside the nasopharyngeal tonsils (adenoids), the paired tubal tonsils and lingual, palatine tonsils are a major component of the so-called Waldeyer's ring (Andersson *et al.*, 1994).

All parts of Waldeyer's ring are indeed strategically located to perform regional immune functions because these structures are exposed to both airborne and alimentary antigens. However, although tonsils play an important immune inductive role as components of mucosal associated lymphoid tissue (MALT), this structure also shows similarities with lymph nodes and may in addition participate as effective organs of local systemic type as well as mucosal-type adaptive immunity (Brandtzeag, 2003).

The blood supply of the palatine tonsil is mainly provided by the tonsillar branch either of the ascending palatine artery or the facial artery. Other small arterial branches originate from the lingual and ascending pharyngeal arteries. The arteries enter the tonsil preferentially at the inferior pole. The lymphatic vessels lead to the submandibular lymph nodes and thereafter to the deep cervical lymph nodes.

The palatine tonsils are covered by a stratified squamous non-keratinizing epithelium, supported by a capsule of connective tissue, containing blood vessels, nerves and lymphatics.

The size of the tonsils is most prominent during childhood and directly proportional to the bacterial load and the number of B and T cells. Later, age-dependent size reduction (tonsillar involution) can be observed (Nave *et al.*, 2001).

2.2: Function and histology of palatine tonsils:

The two oval palatine tonsils are located in the tonsillar fosse between the anterior (Palatine glossal) and posterior (palatine pharyngeal) tonsillar pillars (Richardson, 1999).

Histologically, tonsil tissue consists of four well-defined micro compartments, which all participate in the mucosal immune response: the reticular crypt epithelium, the extra follicular area, the mantle zone of lymphoid follicles, and the follicular germinal center (Van Kempen *et al.*, 2000).

2.2.1: Tonsillar crypts and epithelium:

Human palatine tonsils contain 10-30 blind-ending tubular, branched crypts extending through the full thickness of the organ. The reticulated crypt epithelium is a modified form of the stratified squamous epithelium in which antigens endocytosis by specialized microvilli-possessing epithelial cells (M-Cells) (Sachse *et al.*, 2005).

The non epithelial elements identified in the reticulated epithelium include lymphoid cells, numerous antigens presenting cell (APC), plasma cell, and a network of small blood vessels (Perry, 1994).

Reticulation continues throughout life and presence of nonepithelial cells is considered a physiological character of this epithelium, which is constantly

exposed to airborne alimentary antigens. Any inflammatory changes are superimposed on the pattern of reticulation (Falk and mootz, 1973).

The right balance of the number of epithelial and nonepithelial cells in the reticulated crypt epithelium might be vital for the preservation of efficient mucosal protection. (Brandtzaeg, 1984).

2.2.2: Lymphoid follicles:

Primary follicles of the tonsils consist mainly of nave B cell (IgM + IgD+) and follicular dendritic cell (FDCs). A secondary follicle that results from immune activation can be divided into more or less well-defined morphological compartment (Brandtzaeg, 2003).

The lymphoid follicles zones include the mantle zones and follicular germinal centers. Maturation of B cell Igs and development of B cell memory occur within germinal centers (GCs) and require help provided by CD4+ T-cells (MacLennan *et al.*, 1997). T cells supporting B cell differentiation have been widely accepted to belong to the Th2 subset, because Th2 clones support in vitro antibodies (Abs) production better than Th1 cells do, and also secrete a pattern of cytokines with well documented effects in B cell growth, differentiation, and isotype switching (Abbas *et al.*, 1990).

In contrast, B cells do themselves play an important role in the development and maintenance of CD4+ T-cell memory, and in regulation of Th cell polarization. Although B cells are inadequate APCs for priming naive T cells, they can present antigens (Ags) for primed or memory T cells and thereby elicit cytokine secretion. Under conditions being optimal for induction of strong humoral immune responses, cognate interaction between B and T cells takes place at the edge of the follicles ~2 days after primary immunization and is followed by a GC formation at day 4-5. Thereafter, both B and T cells continue to divide inside the growing GC (Johansson-Lindbom *et al.*, 2003).

Accordingly, follicular CD4+ T cells, initially primed on T cell zone-localized dendritic cells (DCs), are likely to receive a second boost of

differentiation signals during the interaction with GC B cells. We have recently shown that GC B cells produce IL-4 *in vivo*, suggesting that GCs do provide an appropriate microenvironment for Th2 development (Johansson-Lindbom & Borrebaeck, 2002).

2.2.3: Extra follicular region:

The extra follicular regions consist of cellular immunity relating cell (such as interdigitating dendritic cell (IDCs), macrophages and lymphocytes with the helper phenotype, CD4⁺ which consist of both nave CD45RA⁺ and memory CD45RO⁺ subsets and some express the IL-2 receptor (CD25) as a sign of activation), humoral immunity relating cells (Plasma cells), and specialized venules, the so called high-endothelial venules (HEV). The HEV are necessary for the entry of T and B cells from the blood into the tonsils. (Okato *et al.*, 1989).

In the extrafollicular area, a specific mixture of cytokine producing cells (IL-1 α and TNF- α from macrophages as well as IDC, IL-2, IL-4 and IFN- γ from T cells) and antibody production is found (Hoefakker *et al.*, 1993).

The functional interaction between the IDCs with macrophages and lymphocytes was reported to provide the cell with an opportunity for immune regulation at the earliest stage of immune induction (Ozbilgin *et al.*, 2004).

The APCs in the extrafollicular was low (1%) but this number increased under infection condition. Ozbilgin *et al.* (2004) observed the interaction between macrophages and IDCs in the extrafollicular areas by electron microscopy. However, Okata *et al.* (1989) showed the presence of morphological interaction among these cells and suggested that DCs seem to mediate antigen presentation from macrophages to helper T-lymphocytes.

Also, it is known that DCs carry out endocytosis and processing independently (Karchev and Kabakchiev, 1984). Therefore, the aim of these interactions among these cells can be explained as a sign of the regulation of immune responses.

2.3: Mucosal Immunology:

The mucosal surfaces of the respiratory, the gastrointestinal and the urogenital tract, covering a total of 200-300m², are the largest areas within the body in contact with the external environment and thus are major sites of antigens exposure (Tlaskalova-Hogenova *et al.*, 2002).

Discriminating between pathogenic antigens, towards which a protective immune response has to be established, and harmless antigens such as food, airborne antigens and commensal bacterial flora that should be ignored is the most challenging task of mucosal immune system. In order to handle these challenges the mucosal immune system has generated two arms of adaptive defenses: (1) antigen exclusion performed by secretory IgA and Secretory IgM antibodies to modulate or inhibit adherence or colonization of microorganisms and prevent penetration of potentially dangerous antigens. (2) Suppressive mechanisms to avoid local and peripheral overreaction against innocuous substances contacting the mucosal surfaces. The latter arm is referred to as oral or mucosal tolerance (Wiedermann, 2003).

Numerous works in field of mucosal immunity had been done in Babylon University and then started when Shnawa tried to separate gut mucosal immunoglobulin from normal subject in 1992, but the work had not been prepared for publication.

Shnawa and AL-Saadi (2001) separate gut mucosal immunoglobulin and they use it as infectious probe in typhoid patients. Followed by Abd and Shnawa (2002) in comparison between local and systemic humoral immune response in typhoid patient then in chronic ear discharge patients by Addos (2005), in vaginosis women (Abd, 2006), and in patients with meningitis (AL-Thahab, 2006).

Most external mucosal surfaces are replete with organized follicles and scattered antigen-reactive or sensitized lymphoid elements, including B-cells, T

lymphocytes, T-cell subsets, plasma cells, and a variety of other cellular elements involved in the induction and maintenance of immune response. The major antibody isotype in external secretions is secretory immunoglobulin A (sIgA) which secreted in proportion of 40 mg of IgA per Kg of body weight daily (Ogra *et al.*, 2001).

Tonsillar immunoglobulin secretion differs from the usual MALT pattern in which IgG is predominant in palatine and nasopharyngeal tonsils (Scadding, 1990).

2.4: Immunology of palatine tonsils:

Tonsil tissue is located at the get way of the respiratory and alimentary tract and belongs to the MALT (Xie *et al.*, 2004). It serves as a first line of defense against environmental harmful factor, including microbial pathogens (Kasenomm *et al.*, 2005). Protection of the mucosal surface in this area depends on both innate non-specific defense mechanisms and adaptive specific immune reaction (Olofsson *et al.*, 1998).

2.4.1: First step in the immune response:

The upper airway has important mechanism for protection against microorganisms. Phagocytic clearance and airway surface liquid (ASL), which is antimicrobial agents, expressed and secreted by epithelial cell, mucosal surface and sub mucosal glands (Claeys *et al.*, 2003).

Air way surface liquid (ASL) include lysozyme, lactoferrin, secretory leukocyte proteins inhibitor and human B-defense (HBD) these antimicrobial agents co-operate to give an immediate protection against bacterial overgrowth and induce an appropriate local and systemic adaptive answer through their signaling function (Ferine-King *et al.*, 2002 ; Claeys *et al.*, 2003).

When the organism is virulence and the host defense is failed, trapped organisms multiply within and on the tonsil and the Ags are taken up by specialized cell of lymphoepithelium that functionally and ultrastructurly

resemble (M) cells in the peyer's patches .The M cell endocytose Ags and deliver them to underlying macrophages, dendritic cells and lymphocytes. However, the Molecular mechanisms for selective adherence of microorganisms to M cells are poorly understood. (Park *et al.*, 2003).

The complex histological configuration of the epithelia is fundamental for the uptake and presentation of antigen to the sub epithelial immunocompetent cells. This allows the whole organ to act as a functional unit and hence to play an important role in fighting microorganism (Passali *et al.*, 2004).

APCs carry the antigen and present it to the lymphocytes resulting in the induction of an immune response (Ozbilgin *et al.*, 2004). The lymphoid cells found in the space of reticulated crypt epithelium of the human palatine tonsil are mainly B cells (80-90%) and T helper cells (CD4+) (10-20%) (Morente *et al.*, 1992).

The innate component and adaptive component requires the help of soluble mediator, cytokines for their interrelated function (Narin and Helbert, 2002).

The dominating cytokines in tissues from recurrent tonsillitis and tonsillar hypertrophy were GM-CSF, G-CSF, and TGF- β 1-3 which were synthesized predominantly in the reticular crypt site. IL-1 alpha and beta were localized to the surface and crypt epithelium and to scattered regions in the extrafollicular area (Agren *et al.*, 1996).

Quantitative immunohistochemistry reveals that IgG-containing B cells predominate in all lymphoid compartments, including follicles, extra follicular areas and reticular epithelium; whereas IgA cells are found predominantly in extra follicular areas, especially sub epithelial area, and IgM cells are in follicles (Xie *et al.*, 2004).

Unlike the adenoid, there is no tonsillar production of secretory piece, so both IgA and IgG pass out directly in to the pharyngeal secretion by leaking between epithelial cells, this is enhanced when there is inflammation (Scadding, 1990).

2.4.2: Second step in the immune response:

After passing of antigen via micropore (M) cells the Ags are taken up by APC in which processed and present to CD4⁺ T lymphocytes in extra follicular region (Brandtzaeg *et al.*, 1999).

Nave B cells are stimulated and transform in to a centroblast (CB) that proliferates within the dark zone of the GC then the CB developed in to centrocytes (CCs) which composed the basal light zone of GC. In the apical light zone of the GC they differentiated in to plasma cells and memory B cells (Klein *et al.*, 2002). Plasma cells in the GC of both IgG and IgA isotypes, there is, however a predominance of IgG isotype, 60-70% IgG versus 15-20% IgA (Ogra *et al.*, 2001).

Although the number of T cells in the lymphoid follicles is limited, these few cells have a strong influence on the fate of B cells. Since large fraction of GC B cells undergoes apoptosis as they have acquired deleterious somatic mutation in their IgV region, these T cells are capable of expressing several cytokines e.g. IL-4 that inhibit the apoptosis of B cells (Nave *et al.*, 2001).

2.4.3: Role of cytokines in palatine tonsils immune response :

Human tonsils are sites of continuous lymphoid activation and cytokines production, this reflect the constant stimulation by environmental antigens encountered in the oropharyngeal cavity. The information regarding the functional capacity of tonsillar B cells is substantial. However, the analysis of cytokine-producing cells within the microenvironment of this lymphoid tissue may reveal T-cell responses required for primal immunological control .Both Th1 and Th2 types of cytokine responses were expressed in tonsil tissue (Andersson *et al.*, 1994).

Salvi and Holgate (1999) demonstrated that healthy human airway epithelial cells constitutively produce IL-5, a major cytokine implicated in the growth and differentiation of post-switch mIgA + B cells to IgA producing plasma cells. The human airway epithelium also constitutively produces IL-2,

TGF- β , IL-6, and IL-10 factor which are essential for B cells clonal proliferation, IgA isotype switch and differentiation in to IgA-producing plasma cells.

The immunohistochemistry study of tonsils from patients with RT-TE indicated that the chemoattractant cytokine IL-8, was a more prominent monokine in recurrent tonsillitis patient, and the IL-8 synthesizing cells were predominantly found in the crypt epithelium. While IL-6 and IL-10 production cells has a scattered distribution in the extra follicular sites. IFN- γ and TNF- α producing cells was also gathered in the extra follicular area. A section obtained from same patient showing TGF- β 2 containing cells localized in the crypts and germinal centers without activity in the mantel zones (Andersson *et al.*, 1994).

GC B cells as well as FDC failed to express detectable quantities of cytokine like IL-1 α , IL-1 β , IL-2, IL-3, IL-5, IL-6, IL-10, TNF- α and IFN- γ . IL-4 is the only cytokine consistently expressed in GC and may be important for the development of B cells in the GC (Butch *et al.*, 1993).

On the other hand GC T lymphocytes produce IL-2, IL-4, IL-10, IFN- γ and TNF- α in low concentration in contrast to the T cells outside the GC (Toellner *et al.*, 1995).

Holder *et al.*, (1992) ; Mossman and Coffman (1989) show that certain cytokines (TGF- β 1-3) are involved in the regulation of IL-1 production, the Ig switch to all IgG subclass, IgA production, and also in induction of the Bcl-2 gene, thus preventing apoptosis of immune activation B lymphocytes.

Differential production of Th cells during an immune reaction has important regulatory effects on the nature of the immune activation. Responses in mice that are characterized by substantial IFN- γ , TNF- β and IL-12, but little or no IL-4, IL-5, and IL-13 production, that is Th1 responses, result in activation of macrophages to kill intracellular micro-organisms, cytotoxicity, delayed-type hypersensitivity (DTH) and in IgG2a synthesis (Chizzonite *et al.*, 1991 ; Bogen *et al.*, 1993). In contrast the Th2 responses, in which the production of IL-4, IL-5, IL-10, and IL-13 predominates, generate IgG1 and IgE secreting cells preferentially and may induce eosinophilia (Minty *et al.*, 1993 ; Bradding *et al.*,

1993). Human CD4⁺ T cells clones, specific for bacterial antigens on the one hand and allergens on the other, exhibit Th1 or Th2 like cytokine production profiles in line with responses described for mouse Th cells (Romagnani, 1992).

2.5: Oral mucosal tolerance :

The oral mucosa is exposed to a high density and diversity of gram-positive and gram-negative bacteria including commensals and pathogens. Local and systemic immune responses are down modulated and reprogrammed by induction of oral tolerance. Induction of immune tolerance toward commensals combined with responsiveness to pathogens is essential to sustaining immune homeostasis while preventing life-threatening infections. It is unclear how the oral mucosa is able to quickly distinguish commensals from pathogens and mount an appropriate response (Muthukura *et al.*, 2005).

Toll-like receptors (TLRs) are the principle pattern recognition receptors on innate immune cells. TLRs recognize microbial structure and transmit this information into the cell, culminating in an inflammatory cytokine response and co-stimulatory molecule expression involved in induction of adaptive immunity.

TLR2, along with TLR1/6, recognizes gram-positive peptidoglycan while TLR4, along with CD14 and other adaptor molecules, recognize pathogen associated molecular patterns such as lipopolysaccharides (LPS) from gram-negative enteric bacteria (Takeuchi *et al.*, 2002).

S-IgA, IgE, intraepithelial lymphocytes, dendritic cells, T lymphocytes and their subsets, and a wide spectrum of immunoregulatory and proinflammatory cytokines and chemokines available locally in the mucosal tissues. Oral antigen induces T-helper 2 (IL-4 and IL-10), T-helper 3 (TGF- β), T cells plus CD4, CD25, regulatory cells in which induction of oral tolerance is enhanced by IL-4, IL-10, anti-IL-12, TGF- β and anti-CD40 ligand (Faria and Weiner, 2005).

Mucosal immunoglobulin exhibit antibacterial, antiviral, antiparasitic, and antifungal specificity in both in vitro and in vivo settings. Protection against

mucosal infections has been frequently correlated with the presence and level of specific antibody activity existing in the mucosal surface (Ogra *et al.*, 1980).

It appears that an immune response to a protein antigen administered via the mucosal route can be expressed as (1) mucosal immune response with S-IgA antibody, (2) systemic immune responses priming, with development of serum IgG, IgM and specific cellular immunity, and (3) development of mucosal tolerance with systemic immunologic hyporesponsiveness with or without any change in IgA-specific mucosal immune reactivity (Ogra *et al.*, 2001).

The mechanisms underlying the development of mucosal tolerance include direct inactivation of antigen-sensitized lymphocytes via clonal deletion or anergy, interaction between regulatory and effector T cells (CD4+, CD8+, Th1 versus Th2, and $\gamma\delta$ T-cell receptor, tolerogenic proteins, hepatic processing of antigen (Ogra *et al.*, 2001).

2.6: Recurrent tonsillitis :

Recurrent tonsillitis (RT) is a chronic inflammatory process in palatine tonsils occurs when virulence and activity of pathogenic organism in the tonsillar lymphoid tissue exceed the protective potency of activated lymphoid and immunoglobulin-producing cells (Scadding, 1990). A leading therapeutic approach for such a condition has been tonsillectomy (Davidson and Calloway, 1980).

The surface and deep bacterial flora of recurrently inflamed tonsils consist of an abundance of potentially pathogenic aerobic and anaerobic bacteria (Kasenomm *et al.*, 2005).

The predominant anaerobic isolates were *Bacteroides sp.*, *Fusobacterium nucleatum*, anaerobic gram positive cocci and *Eubacterium sp.* While the predominant aerobic isolates were alpha-hemolytic, *Staphylococcus aureus*, beta-hemolytic streptococci, and *Haemophilus sp.* (Brook *et al.*, 1980).

Staphylococcus aureus and *Streptococcus pyogenes* are recognized as the common tonsillitis causing pathogens and are especially well known for its

tendency to cause chronic infection (Abhay *et al.*, 2005; Brook and shah, 2001; Brook *et al.*, 1981; Brook *et al.*, 1995).

2.6.1: Staphylococcus aureus:

Staphylococcus aureus (*S.aureus*) is a major human and animal pathogen. During infection, this organism is not able to attach to and enter host cells by using its cell surface-associated factors only but also exports toxins to induce apoptosis and kill invaded cells (Liang *et al.*, 2006).

The virulence factor include both structural components of bacterial cell wall, such as peptidoglycan and lipoteichoic acid, and surface proteins including protein A, fibronectin-binding protein and clumping factor, while secreted proteins include invasins (that promote bacterial spread in tissue such as leukocidin, staphylokinase, hyaluronidase), membrane-damaging toxins (that lyses eukaryotic cell membranes such as hemolysins, leukotoxin and leukocidin) and tissue-damaging toxins (such as enterotoxin A-G, toxic shock syndrome-toxin (TSST-1), and V8 protease(Kielian *et al.*, 2001).

Plitnick *et al.*(2001) Study the role of lipoteichoic acid in dampening the host immune response to gram-positive organisms and prolonging bacterial survival via inhibition of IL-2 function.

Staphylococcal protein A, comprise 7% of the cell wall, bind the CH2 and CH3 domains of the FC fragment of IgG2 and IgG4. Thus, not only does protein A compete with the FC receptors of phagocytes for antibody molecules, but also coats staphylococci with host protein and therefore hides them from phagocytes and the cells responsible for immunity (Navarry and Schneewird, 1999 ; Gao and Stewart, 2004).

Previous studies identify alpha-Toxin as a key virulence determinant for the survival of *S.aureus* intracellularly, in both epithelial cell and neutrophils, providing a means by which the organism may establish chronic infection (Greshma *et al.*, 2000 ; Kahl *et al.*, 2000).

Moreover, Panton-valentine leukocidin (Luk-pv) from *S.aureus* V8 strains is important for the pathogenicity of staphylococcal disease in which Luk-pv induce polymorph nuclear neutrophils (PMN) to generate leukotriene B4 (LTB4), which play a crucial role as an inflammatory chemotactic factor for neutrophils (Konig *et al.*, 1994).

The bacterial immune suppression, intracellular survival of *S.aureus* and production of virulence factor most probably play an important role in complex response to *S.aureus* in the host (Kielian *et al.*, 2001).

2.6.2: Streptococcus pyogenes:

It's an important human pathogen, with the ability to cause a variety of infection including streptococcal pharyngitis, one of the most common bacterial infections in childhood (Cywes *et al.*, 2000).

Group A streptococci (GAS) possess a large repertoire of the known and suspected virulence factors, some of those factors may be required for successful adherence and colonization of the host at various sites (e.g., lipoteichoic acid, capsule, and protein F). Some may protect GAS from constitutive host defenses (e.g., M protein, hyaluronic acid capsule, Protein G, Streptococcal inhibitor of complement, and C5a peptidase still others may function primarily to destroy and allow streptococci to spread through the host (e.g., streptolysin S, streptokinase, streptodornase and hyaluronidase) (Heath *et al.*, 1999). Experimental infection studies in mice have shown that mutant strains of GAS deficient in capsular polysaccharide fail to cause infection after intranasal inoculation, because the capsular polysaccharide forms an outer most layer on the bacterial surface, it is likely to be important in adhesive interactions between the bacterial cell and pharyngeal epithelium (Cywas *et al.*, 2000).

Certain M protein serotypes, such as M types 1,3,5,6,14,18,19, and 24 of *S.pyogenes*, are found associated with throat infection and rheumatic fever. It is well established that group A streptococci are antiphagocytic due to surface exposed M protein and hyaluronic acid capsule (Cunningham, 2000).

S.pyogenes secretes a number of proteins that inhibit immunoglobulin mediated opsonophagocytosis e.g. Endos, hydrolyzes the conserved N-linked Oligo Saccharides on the heavy chains IgG, and streptococcal cysteine proteinase also called IdeS, cleaves IgG into FC and monomeric Fab Fragment (Collin *et al.*, 2002).

It is well documented that GAS express on their surfaces streptococcal C5a peptidase (SCP), this enzyme inactivate C5a and destruct its ability to act as an anaphylatoxin and a strong chemoattractant thus, SCP is considered an important virulence factor of pathogenic streptococci (Koroleva *et al.*, 2002).

2.7: Effect of tonsillectomy on immune system:

Tonsillectomy procedures are among the oldest surgical procedures still performed. Otolaryngologically, the two main indications for tonsillectomy are upper airway obstruction due to tonsillar hypertrophy and recurrent acute or chronic tonsillitis (Xie *et al.*, 2004).

The effect of tonsillectomy on immune system include the following decreased the levels of serum IgG and salivary secretory IgA, especially in children, for up to 3 years after operating but this alteration do not increase in the opportunity for infections post operatively in compared to an age-matched control group (Bock *et al.*, 1994).

Unal *et al.* (2002) Indicated that children with chronic tonsillitis show significant higher level of IL-1 β and IL-6 in compared to healthy controls in pre-operative period. After the tonsillectomy IL-18 and IL-6 levels were significantly reduced.

Recent study demonstrated that children with chronic tonsillitis have increased levels of CD19 and CD23 B lymphocytes. After tonsillectomy, CD8 T lymphocyte level increased and B cell levels returned to normal (Ikinciogullari *et al.*, 2002). In addition, tonsillectomy has no effect on air way surface liquid except lactoferrin, which declined significantly (Kirstila *et al.*, 1996).

3. Material and Methods:

3.1: Patients:

The study involved 103 recurrent tonsillitis patients (age range 5-30, 61 females and 42 males) selected among 180 patients referred to Rizgary Teaching Hospital in Erbil city for tonsillectomy from January 2006 to January 2007. Patient was selected from the operation list on two or three particular days of the week.

Each patient had a history of tonsillitis episodes for at least one year, characterized by sore throat or swollen painful tonsils with fever or symptoms of systemic illness, but absence of symptoms of possible viral upper respiratory tract infection, such as running nose and cough.

patients included in this study were children, puberty, adolescents, and young adults in which classified into four age groups (5-9 years, 10-14 years, 15-19 years, and 20-30 years)(Lam, 2003 ; Breslin *et al.*, 2003 ; Carla *et al.*, 1994). Most of them from low socioeconomic state.

A questioner form were provided to each patients to inquire about important point relevant to the study as shown in table (3-1)

3.2: Normal controls:

The controlling group consisted of 40 volunteers from same urban population and was matched for age to the 4 age group of patient, female, males, who were not suffering from recurrent tonsillitis episodes.

Table 3.1: Medical Data Form.

Name			Tel.
Age			
Sex	Male	Female	
Indication for tonsillectomy -Recurrent acute tonsillitis -Peritonsillar abscess -Tonsillar hypertrophy with sleep disturbance			
On examination -State of tonsils -Pharyngitis(+ve)	Hypertrophy	Atrophy	
-Pharyngitis(-ve)			
Drug history			

3.3: Procedure used in this study:

3.3.1 Stains:

3.3.1.1- Gram's stain

It was prepared according to Hucker modification (Atlas *et al.*, 1995).

3.3.1.2- Wright's stain

It was prepared by grinding 0.1gm of Wright powder in a mortar while slowly adding small amounts of absolute methanol to a final volume 60ml and allowed to stand at room temperature for 1-2 days then filtrated before use. Stored in tightly Stoppard bottle to prevent evaporation. This stain was used in leukocyte stain for phagocytic activity. (Metcalf *et al.*, 1986).

3.3.1.3- Harris hematoxylin and eosin solution

It was prepared according to Luna, 1968.

3.3.2: Reagents:

The reagents were prepared accordance to (Cruickshank *et al.*, 1975). Thy were used in diagnosis of bacterial isolates.

3.3.2.1- Oxidase reagent

Prepared by dissolving 1g tetramethyl-p-phenylenediamine dihydrochloride, (BDH) in 100ml distilled water (D.W).

3.3.2.2- Catalase reagent

This reagent was prepared by adding 3ml hydrogen peroxide (H₂O₂) to 97 ml D.W. the final concentration 3% (Cruickshank *et al.*, 1975).

3.3.2.3- Nitroblue tetra zolium (NBT)

One NBT tablet (purified C₄₀H₃₀ Cl₂N₁₀O₆ from Sigma-Aldrich, St. Louis, Germany) was dissolved in 1ml of deionized water then complete the volume to 10ml with phosphate-buffered saline to obtain concentration of 0.1% and then filtered with 0.22 µm pore size (Albert filter paper) according to manufacture instruction.

3.3.3: Solutions

3.3.3.1- Normal saline

It was prepared by dissolving 0.85g NaCl (BDH Company, molecular weight 58.44) in little amount of D.W and was complete to 100ml.

The solution was sterilized by autoclaving at (121C°, 15 pound for 15 minutes). This solution was used in formal saline (Garvey *et al.*, 1977).

3.3.3.2- Formal saline

This solution was prepared at 0.5% concentration by adding 0.5ml formaldehyde (H-CHO) BDH, to 99.5ml normal saline which was used as solvent to tonsils mucosal immunoglobulin (TIgs) (Kwapinski, 1972).

3.3.3.3- Tris buffer solution

It was prepared by dissolving 12 g tris material (NH₂C (CH₂OH) ₃). Taab Company, in a small amount of D.W, and was completed to 1 liter with D.W, then PH was adjusted to 7 by using HCl 0.1N, this solution was used for preparing polyethylene glycol (Johnston and Thorpe, 1982).

3.3.3.4- Polyethylene glycol (PEG6%)

Six g polyethylene glycol ($\text{HO}(\text{C}_2\text{H}_4\text{O})_n\text{H}$) (BHD, M.W.6000) was dissolved in a small amount of tris solution as indicated in the volume was completed to 100ml with tris solution and the PH was adjusted to 7.4 this solution was used for TIgs separation (Johnston and Thorpe, 1982).

3.3.3.5- Phosphate buffered solution

This solution was prepared by dissolving 22.9 g potassium phosphate (KH_2PO_4 , BDH) and 19.5g Di-Potassium hydrogen phosphate (K_2HPO_4 , BDH) in 1liter of D.W. and the PH was adjusted to 7.2 by using KOH. This solution was used for preparation of Hepes-BSS (Myers, 1995)

3.3.3.6- Hepes – Buffered Balance Salt Solution (Hepes – BSS):

This buffer was prepared by adding the above stock solution in the following proportions : 5ml of Magnesium sulphate - $7\text{H}_2\text{O}$ ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) (41.3 g/l), Fluka), 15ml of calcium chloride (CaCl_2 (12.45g/l), BDH), 20 ml of Potassium chloride (KCl (12.5g/l), BDH) , 605 ml of sodium chloride (NaCl (9.83 g/l), PEAXNM), 30 ml of Hepes buffer [prepared by adding 80g of hepes ($\text{C}_8\text{H}_{18}\text{N}_2\text{O}_4\text{S}$ >99.5%, Sigma, USA) and 13.4 g of sodium hydroxide (NaOH , Alpha, India) to 1 liter of D.W., and the PH was adjusted to 7.2 by HCl or NaOH]. And 10 ml of phosphate buffer. This solution was used as buffer in E-rosette test. (Myers, 1995).

3.3.3.7- Alsever's solution (pH=6.1):

The solution was prepared by dissolving the following materials in 1200 ml D.W.; 24.06 g of glucose (BDH), 9.6 g sodium citrate (BDH), and 5.04 g sodium chloride (BDH), the result solution was sterilized by filtration through 0.22 μm pore size and was used as preservative for sheep red blood cells (sRBC) (Garvey *et al.*, 1977).

3.3.3.8- Benzal Konium Chloride (1/1000):

The solution was prepared by adding 0.2 ml of Alkyl benzyl dimethyl ammonium chloride (C₂₂H₄₀ClN, conc. 50%, samara) to 99.8 ml D.W. This solution was used in preparation of gram positive bacterial antigen (McCoy and Kennedy, 1960).

3.3.3.9- Wright stain's buffer:

This buffer was prepared by adding 6.63g potassium phosphate, monobasic (KH₂PO₄, BDH) and 2.56g of sodium phosphate, dibasic (anhydrous Na₂HPO₄, BDH) to 1 liter of D.W. This buffer was used for washing the slid stained with Wright stain.

3.3.4: Media:**A- Bacteriological media.**

- 1- Blood Agar (Oxoid, England): was prepared according to manufacture instruction.
- 2- Brain Heart Infusion Broth (BHIB) (HIMEDIA, India): was prepared according to manufacture instruction.
- 3- Brain Heart Infusion Agar (BHIA) (HIMEDIA, India): was prepared according to manufacture instruction.
- 4- Mannitol Salt Agar (Oxoid, England): was prepared according to manufacture instruction.

B- Immunological media.

- 1- Agar-agar (HIMEDIA): This medium was prepared by dissolving 1.5g agar in 100 ml D.W. and sterilized by autoclave and poured in to sterile glass Petri dish .This media was used for migration of leukocyte.
- 2- Basal Medium Eagle (Sigma-Aldrich): this medium was prepared by dissolving 1g of Eagle medium in 100ml D.W. and then sterilized by membrane filtration 0.22µm pore size .It was used as nutritive media for leukocyte in leukocyte inhibitory assay (Soborg, 1969).

3.4: Blood sample:

Serum and heparinized blood specimens were collected before tonsillectomy. 5ml of blood was collected from each patient using sterile disposable syringes. 3ml was put in to AFMA-disposable tubes, without anticoagulant, then the serum was collected after centrifugation at 2500 r.p.m. for 5 minutes and it was stored at -20C°.

The other 2ml of blood was put in FAMA-disposable tube with anticoagulant for phagocytosis, LIF and E-rosette test as shown in figure (3.1).

3.5: Tonsillar specimens:

After excision, the tonsils was placed in sterile Petri dish and taken immediately to the microbiological laboratory.

Under strict aseptic condition the tonsils were section in half after heat searing of the surface and the core material was cultured in BHIB for 8-10 hr. Preparing for further bacteriological study, and the tonsillar tissue were cut and minced with cold normal saline and homogenized with proportion of 2 ml of cold normal saline for 1 gm of tonsillar tissue. 3ml of tonsil suspension was used for E-rosette and LIF test.

Immunoglobulin titer was determined by centrifugation 5ml of tonsillar suspension for 30min at 3500 r.p.m. then equal volumes of PEG 6% were added to supernatant and left over night at 4C then centrifuged for 15min. at 3500 r.p.m., the pellet was dissolved in formal saline and this represent TIgs. And then 0.1 ml of TIgs was added to 0.9 ml of N.S and used for titration.

The remaining tonsils suspension was centrifuged at 3000 rpm for 10 min. for determination of mucosal cytokine as shown in figure (3.2).

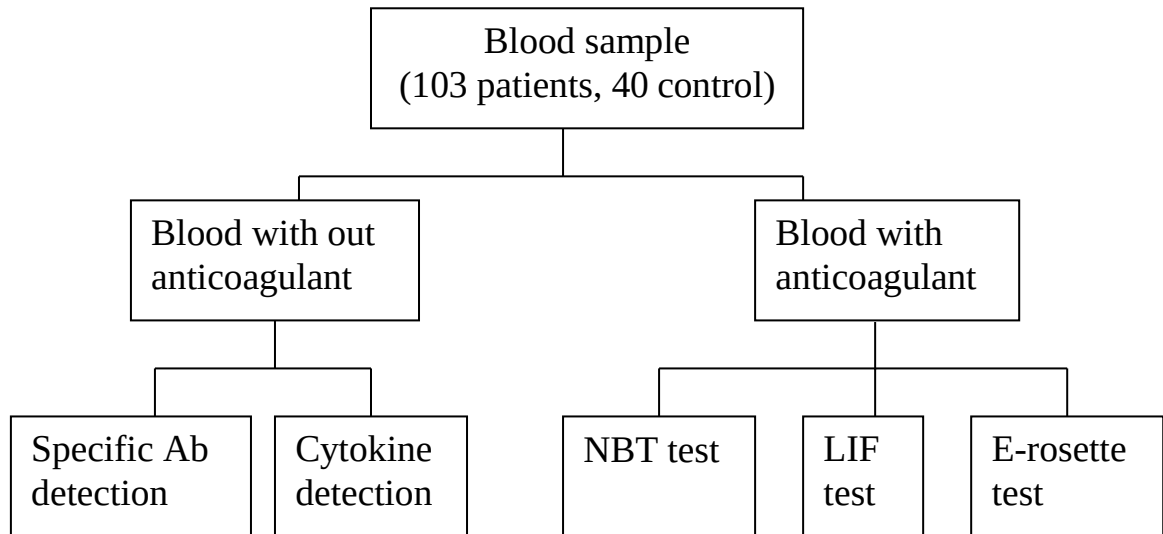


Figure 3.1: Study of Systemic immunity.

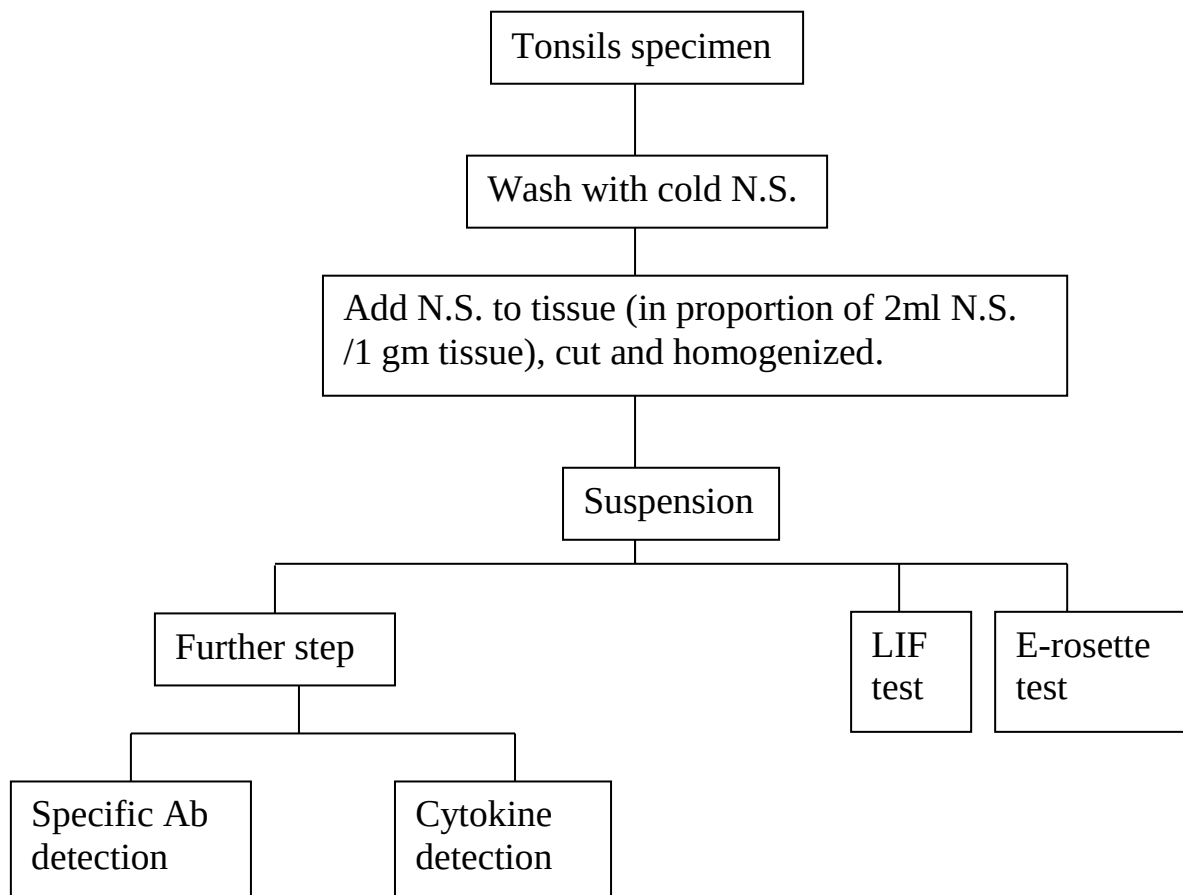


Figure 3.2: Study of Mucosal immunity.

6: Bacteriological investigation:

3.6.1: Culture

The swab in BHIB, which taken from tonsil specimen, were cultured on both blood agar plates and examined after overnight incubation at 37C°, as the chocolate plates were incubated in candle jars (Reilly *et al.*, 1981).

3.6.2: Identification

The clinical isolates were primarily identified on basis of colony characteristics, gram stain morphology, blood hemolysis, catalase, oxidase, and mannitol fermentation. Then *S.aureus* and *S.pyogenes* was detected by using Api staph. And Api 20 strep. according to manufacture instruction (BioMerieux).

3.6.3: Cell free culture antigen (CFC):

The bacteria (*S.aureus*, *S.pyogenes*, mixed bacteria) were cultivated primarily on blood agar then in BHIB. Over night culture were harvested.

The number of bacteria was reduced by double centrifugation at 5000 r.p.m for 15min .Then the supernatants were filtered with 0.45µm pore size filter to gain a sterile solution. Evidence of sterility was performed by cultivation of sterile supernatant on blood agar overnight with no growth(Sachse *et al.*, 2005).The supernatant was used as sensitizer in leukocyte migration study.

3.6.4: Cell surface antigen:

The cell surface antigen of gram positive bacteria was prepared according to McCoy and Kennedy, 1960.

- 1- The isolated bacteria were cultured on blood agar for 24 hr. at 37C°.
- 2- 6ml sterile N.S. was added to culture and bacterial growth as harvested.
- 3- Suspension was collected in sterile tube then mixed with vortex for 3 min.
- 4- 5ml of suspension was centrifuged at 4000 r.p.m for 5min.

- 5- The supernatant was discarded and the pellet was washed with 5ml N.S. and then centrifuged at 3500 r.p.m for 5min, The supernatant was discarded.
- 6- 5ml of benzile konum chloride (0.001%) was added to pellet and mixed with vortex.
- 7- 10 international units of antigen suspension was prepared by adding benzile konum chloride (0.001%) to the suspension which compare with 10IU opacimeter (W.H.O international reference).
- 8- The 10IU of antigen suspension was mixed in vortex and then incubated at 37C° for half hours.
- 9- Sterility was performed by cultivation of antigen suspension on blood agar overnight with no signs of bacterial growth.

3.7: Immunological investigation:

3.7.1: NBT dye reduction test:

NBT test was performed by the method of Park *et al.* (1968), with the subsequent modification of Freeman and King (1972a). 0.1ml of blood was mixed with 0.1ml of NBT solution in a well of micro titer plate. The mixture was mixed gently and covered to ensure humidity, and incubate at 37C° for 15min. follow this with an equal period at room temperature.

Smear was then made and immersed with Wright stain and left for 5min. then rinsed with Wright stain buffer and allowed to dry. The slide was examined under oil immersion. 100 PMN was counted and then percentage of PMNs reduce NBT was recorded.

3.7.2: Leukocyte migration studies:

The leukocyte migration studies were done according to method of Soborg and Bertram, (1968).

- 1- Two capillary tubes was filled with heparinized blood sample (or tonsils suspension in case of mucosal immunity) and sealed with sterile clay.
- 2- The capillary tubes were centrifuged in haematocrite centrifuge for 5min. (1.5 min. in case of tonsils suspension).
- 3- The capillaries were broken at the cell-liquid interface.
- 4- These cutted capillaries were applied to agar-agar well in Petri dish.
- 5- 50µl basal medium eagle were added to each well.
- 6- 50µl of cell free culture antigen was added to first well and 50µl N.S. was added to control well.
- 7- The plate was incubated in humid jar, in upright position, overnight at 37C°.

The migration was read with and without sensitizer under light microscope using ocular micrometer to read the distance of migration.

The average of migration area with sensitizer (Mz) was related to the average of migration area in control (Mo) and expressed as follows:

$$\text{Migration index} = \text{Mz}/\text{Mo}$$

7.3: E-rosette test:

1- Lymphocyte preparation:

Two ml of heparinized blood was gently mixed with 4ml of hepes-BSS, the diluted blood was carefully added to the 2ml lymphoprep, (Fresenius kabi Norge AS, Oslo, Norway), along side the inner edge of the tube, and centrifuge at 4000 r.p.m for 15min. The leukocytes at the interface were aspirated and washed twice in hepes-BSS then suspended in the same buffer. The cell suspension was used for test performed (Myers, 1995).

2- The E-rosette test was done according to the method of Aiuti *et al.* (1973).

Sheep red blood cell (sRBC) was stored at 4°C in Alsever's solution and used for 1 week. Before use, the cell were washed twice and adjusted to a 0.5% suspension in N.S. The sRBC cell suspension (0.25ml) was mixed with (0.25ml) of lymphocyte suspension and incubated at 37°C for 15min. The mixed cell suspension was spun at 1000 r.p.m for 5min. and then incubated overnight at 4°C. The supernatant fluid was removed, and the top layer of the pellet was gently resuspended by shaking. One drop of the cell suspension was mounted on to a glass slide, covered by cover slip. Two hundred lymphocytes were counted, and all of the lymphocytes binding more than 3 sRBC were considered positive.

Similar method was used to detect E-rosette formation of mucosal lymphocytes.

3.7.4: Detection of specific antibodies:

Specific antibody was detected in both tonsillar suspension (mucosal immunity) and serum (systemic immunity) through tube agglutination test.

1- Eleven clean agglutination test tube were used and 0.9ml N.S. was added to first tube and 0.5ml was added to the rest tubes for serum titration and the other 11 tubes for TIgs titer.

2- 0.1ml of serum was added to first tube and mixed gently by Pasteur pipette and then 0.5ml was transferred to the second tube until 10th tube. Tube number eleven was considered as a control, tube containing 0.5ml N.S., also 0.1ml of TIgs extraction was added to first tube and then 0.5 ml was transferred to the second tubes until 10th tubes. Tube number eleven was considered as a control.

3- 0.5ml of cell surface antigen was added to each tube of serum and 0.5ml of same antigen was added to TIgs tubes.

4- The content was incubated at 37C° for 24hrs.

5- The titer was recorded.

3.7.5: Cytokines Detection:

In group of 30 children (10 children harbors *S.aureus* in their tonsils, 10 children harbors *S.pyogenes* in their tonsils, and 10 children harbors mixed bacteria in their tonsils) and 8 normal control subject the levels of interleukin-6, interleukin-10, and IFN-gamma were determined in the serum as well as the tonsillar tissue supernatant with quantitative enzyme-linked immunosorbent assay.

The lowest detectable values in the standard curve, according to the Beckman coulter company were as follows: interlukin-6, 3pg/ml; interlukin-10, 5pg/ml; and interferon-gamma, 0.08IU/ml.

3.7.5.1: Interlukin-6(IL-6) enzyme immunoassay:

This test was achieved according to the manufacturing company (Immunotech. A Beckman Coulter Company) as follows:

1- The component of the kit equilibrates at room temperature before use.

2-The component of the kit were prepared as the follows:

Reagents	Preparation
Plat : 96 wells	Ready to use
Standard (Lyophilized)	Add volume of distilled water stated on the vial label. This will result in a 10 ng/ml IL-6 solution.
IL-6 Acetylcholine esterase conjugate (Lyophilized)	Add volume of distilled water stated on the vial label.
Diluent 1 IL-6	Ready to use
Diluent 2 IL-6 (Lyophilized)	Add volume of distilled water stated on the vial label.
Washing solution	Dilute 50ml in 950ml of D.W
Substrate (Lyophilized)	Add volume of distilled water stated on the vial label.
Stop solution (Tacrine)	Ready to use

3- The standard solution and the appropriate diluents (Diluent 1 IL-6 for tissue supernatant, diluent 2 IL-6 for serum sample) were prepared to form standard curve for equation calculated. Series of dilution was prepared as follows:

Standard conc. (Pg/ml)	IL-6	Diluent (μ l/ml)
1000	50 μ l of 10 ng/ml calibrator	450
333	150 μ l of 1000 pg/ml calibrator	300
111	150 μ l of 333 pg/ml calibrator	300
37	150 μ l of 111 pg/ml calibrator	300
12.3	150 μ l of 37 pg/ml calibrator	300
0	-	300

4- The protocol of ELISA was included in three steps.

Step 1

- 100 μ l of standard, normal control, and patient sample was added for wells.
- 100 μ l of IL-6 ACE conjugate was added per wells.
- The wells were incubated for 2hr. at 18-25C° while shaking (350 rpm).
- The wells were washed (manual procedure by repeating at least three cycles by turning the plate upside-down and shaking vigorously over the sink, at the end cycle the inverted microtiter plate firmly tap on to a clean absorbent paper.

Step 2

- 200 μ l of substrate was added to each well.
- The wells incubated 30 min at 18-25C° in dark, while shaking (350 rpm).

Step 3

- 50 μ l of stop solution was added to each well.
- The plate absorbance was read at 405nm by Beckman coulter reader.

3.7.5.2: Interlukin-10 (IL-10) enzyme immunoassay:

The test was prepared according to the Beckman Coulter Company.

1- The component of the kit equilibrates at room temperature before use.

2-The component of the kit were prepared as the follows:

Reagents	Preparation
Plat : 96 wells	Ready to use
Standard (Lyophilized)	Add volume of distilled water stated on the vial label. This will result in a 20 ng/ml IL-10 solution.
Biotinylated monoclonal Ab	Ready to use
Diluent	Ready to use
Streptavidin-HRP conjugate	Ready to use
Washing solution (20x)	Ready to use
Substrate	Ready to use
Stop solution(2N sulfuric acid)	Ready to use

3- The standard solution and the appropriate diluents were prepared to form standard curve for equation calculated. Series of dilution was prepared as follows:

Standard conc. (Pg/ml)	IL-10	Diluent (μ l/ml)
2000	50 μ l of 20 ng/ml calibrator	450
400	100 μ l of 2000 pg/ml calibrator	400
80	100 μ l of 400 pg/ml calibrator	400
16	100 μ l of 80 pg/ml calibrator	400
0	-	400

4- The protocol of ELISA was included in three steps.

Step 1

- 50 μ l of standard, normal control, and patient sample was added to wells.
- The wells were incubated for 2hr. at 18-25C° with shaking (350 rpm).
- The wells were washed (manual procedure by repeating at least four cycles by turning the plate upside-down and shaking vigorously over the sink, at the end cycle the inverted microtiter plate firmly tap on to a clean absorbent paper.

Step 2

- 50 μ l of biotinylated antibody was added to each well.
- 100 μ l of streptavidin-HRP conjugate was added to each well.
- The wells were incubated for 30 min at 18-25C° while shaking (350 rpm).

Step 3

- 100 μ l of substrate was added to wells.
- The plate was incubated for 15 min at 18-25C° while shaking (350 rpm).
- 50 μ l of stop solution was added to each well.
- The plate absorbance was read at 450nm by Beckman coulter reader.

3.7.5.3: Interferon-gamma (IFN- γ) enzyme immunoassay:

The test was prepared according to the Beckman Coulter Company.

1- The component of the kit equilibrates at room temperature before use.

2-The component of the kit were prepared as the follows:

Reagents	Preparation
Plat : 96 wells	Ready to use
Standard (Lyophilized)	Add volume of distilled water stated on the vial label. This will result in a 250 IU/ml IFN-gamma solution.
Biotinylated monoclonal Ab	Ready to use
Diluent	Ready to use
Streptavidin-HRP conjugate	Ready to use
Washing solution (20x)	Ready to use
Substrate	Ready to use
Stop solution(2N sulfuric acid)	Ready to use

3- The standard solution and the appropriate diluents were prepared to form standard curve for equation calculated. Series of dilution was prepared as follows:

Standard conc. (IU/ml)	IFN-gamma	Diluent (μ l/ml)
25	50 μ l of 250 IU/ml calibrator	450
6.25	100 μ l of 25 IU/ml calibrator	300
1.56	100 μ l of 6.25 IU/ml calibrator	300
0.39	100 μ l of 1.56 IU/ml calibrator	300
0	-	300

4- The protocol of ELISA was included in three steps.

Step 1

- 50 μ l of standard, normal control, and patient sample was added to wells.
- The wells were incubated for 2hr. at 18-25C° with shaking (350 rpm).
- The wells were washed (manual procedure by repeating at least four cycles by turning the plate upside-down and shaking vigorously over the sink, at the end cycle the inverted microtiter plate firmly tap on to a clean absorbent paper.

Step 2

- 50 μ l of biotinylated antibody was added to each well.
- 100 μ l of streptavidin-HRP conjugate was added to each well.
- The wells were incubated for 30 min at 18-25C° while shaking (350 rpm).

Step 3

- 100 μ l of substrate was added to wells.
- The plate was incubated for 20 min at 18-25C° while shaking (350 rpm).
- 50 μ l of stop solution was added to each well.
- The plate absorbance was read at 450nm by Beckman coulter reader.

3.8: Histological investigation:

Fresh, human palatine tonsils were obtained from routine surgical specimens, corresponding to 15 cases (mixed sex, 5-25y) of recurrent tonsillitis, immediately after tonsillectomy each specimen was processed for routine histopathological examination.

S.aureus was the causative agent of 5 specimens, *S.pyogenes* for 5 specimens, and mixed bacteria for the other 5 specimen.

3.8.1: Paraffin method:

All specimens were fixed in 10% formal saline, washed in saline, dehydrated, cleared and embedded in paraffin wax according to routine techniques of light microscopy.

Slices 5µm thickness were stained with hematoxylin and eosin (H+E) (Morente, 1992).

3.9: Statistical analysis:

Data were processed and analyzed with the statistical package SPSS. Results are expressed as (mean $\pm$ S.E.). Statistical differences of histological parameters were determined by student-newman-keuls test for multiple comparisons after ANOVA .While Duncan test was used to determine differences in phagocytosis, leukocyte inhibition factor(LIF), E-rosette, Immunoglobulin titers, and cytokines after ANOVA. Paired t-test was used to analyze the differences between systemic &mucosal immune response of the RT-TE harbors *S.aureus* or *S.pyogenes* or mixed bacteria. Independent-Samples T test was used to compare patient and control systemic LIF. P-values below 0.05 were considered to be statistically significant.

4. Results:

4.1. Bacterial profile:

The tonsillar microflora of 180 recurrent tonsillitis patient admitted for tonsillectomy was studied. *Haemophilus influenzae* was the most common gram-negative bacteria (23.3%) isolated from the core of the tonsil followed by mixture of *S.aureus* and *S.pyogenes* (22.2%).

S. aureus was isolated in 32 cases (17.7%), while *S.pyogenes* isolated from 31 cases (17.2%), the next common mixture was *H.influenzae* with *S.aureus* which found in 23 cases (12.7%). Other undefined gram positive and negative bacteria constitute 6.6% as shown in table (4.1).

Table 4.1: Profile of bacteria isolated from core of the tonsils.

Bacteria	Number	Percentage (%)
<i>S.aureus</i>	32	17.777
<i>S.pyogenes</i>	31	17.222
Mixed bacteria <i>S.aureus</i> + <i>S.pyogenes</i>	40	22.222
<i>H.influenzae</i>	42	23.333
<i>H.influenzae</i> + <i>S.aureus</i>	23	12.777
Other	12	6.666
Total	180	100 %

4.2. Assessment of phagocytosis:

Nitroblue tetrazolium (NBT) reduction dye was used to assess phagocytic activity of polymorph nuclear phagocytic cell (PMNs). The reduction was graded on the basis of the number on neutrophils with intracellular deposit of formazan stippling per 100 neutrophils counted i.e. percent positive (Fig.4.1) .

This test was used to analyze the possible differences in phagocytic function of the cells in RT-TE with different bacterial cause include *S.aureus*, *S.pyogenes*, and mixed bacteria.

Analysis of variance and Duncan-test revealed that type of bacteria isolated from core of tonsils had the same effect on phagocytosis of PMNs in peripheral blood (Table 4.2).

As illustrated in table (4.3), Age had no significant effect on percent PMN positive for NBT in peripheral blood of RT-TE harbors different bacteria in their tonsils. While the control group show significant increase ($p < 0.05$) in phagocytosis by the age 20-30 years in compared with the first age group.

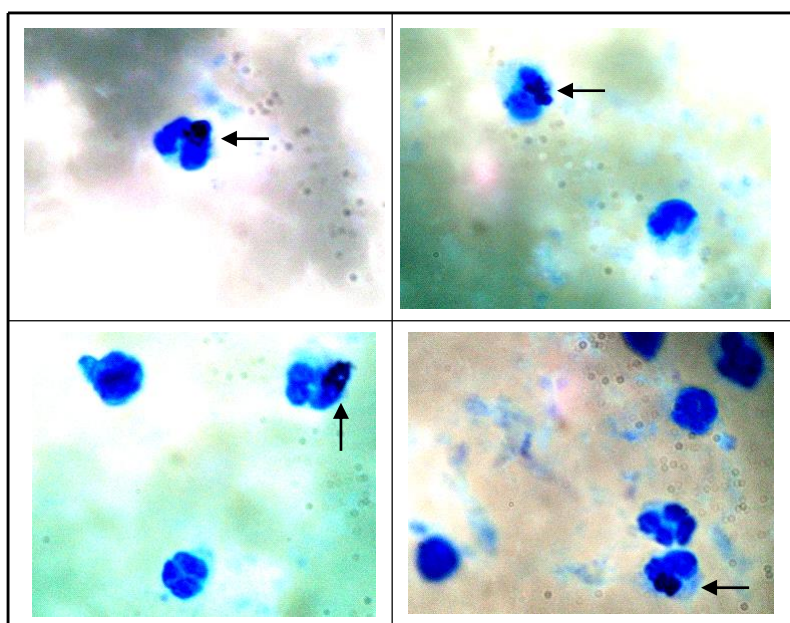


Fig.4.1: Several neutrophils show a typical phagosome type of deposit. Wright stain, 1000x.

Table 4.2: Percent PMN positive for NBT in peripheral blood of RT-TTE harbors *S.aureus*, *S.pyogenes*, and mixed bacteria in their tonsils.

Age (years)	Number of Cases	%NBT-Positive PMNs in RT-TE	
		<i>S.aureus</i>	
5-9	52	9.6135 ± 2.2094	
10-14	34	4.4263 ± 1.1608	
15-19	27	4.7492 ± 1.0280	
20-30	30	6.3623 ± 1.6928	
P<0.05 is significant			

		Controls	P-value
<i>S.pyogenes</i>			
	7.0877 ± 2.2412	4.3070 ± 1.2051	0.441
	10.436 ± 3.2565	4.8871 ± 1.3108	0.131
	8.1053 ± 2.0671	6.1414 ± 1.4175	0.596
	12.356 ± 5.2488	10.036 ± 2.8662	0.537

Table 4.3: Effect of age on percent PMN positive for NBT in RT-TE harbors *S.aureus*, *S.pyogenes*, or mixed bacteria in their tonsils.

RT-TE harbors	Number of Cases	Age(years)				P-value
		5-9	10-14	15-19	20-30	
<i>S.aureus</i>	32	9.6135 $\pm$ 2.2094	4.4263 $\pm$ 1.1608	4.7492 $\pm$ 1.0280	6.3623 $\pm$ 1.6928	0.276
<i>S.pyogenes</i>	31	7.0877 $\pm$ 2.2412	10.436 $\pm$ 3.2565	8.1053 $\pm$ 2.0671	12.356 $\pm$ 5.2488	0.643
Mixed bacteria	40	9.3735 $\pm$ 3.0189	8.6256 $\pm$ 2.0626	5.8460 $\pm$ 1.3666	6.0626 $\pm$ 2.0668	0.767
Controls	40	4.3070 $\pm$ 1.2051	4.8871 $\pm$ 1.3108	6.1414 $\pm$ 1.4175	10.036 $\pm$ 2.8662	0.134

P<0.05 is Significant

4.3. Leukocyte migration studies:

The basic principle behind all migration systems is the release of lymphokines from stimulated lymphocyte, which inhibits or stimulates the migration of target cell.

The numerical value of the migration index expresses whether the migration has been stimulated or inhibited by the antigen. An index greater than 1 means stimulation and less than 1 inhibition of the cell migration.

Systemic LIF in RT-patient shows an inhibition in the first, second, and third age group for all bacteria except for *S.pyogenes* in puberty and mixed bacteria in adolescent group. While adult group shows stimulation for all bacteria (Table 4.4).

On the other hand, systemic LIF for control group gives an index around 1.00 except for *S.pyogenes* in the first and second age group and mixed bacteria in the fourth age group (Table 4.5).

Comparison of systemic LIF between RT-patient and control groups was observed using independent-sample T test. RT-patient shows inhibition in comparison to control groups (Table 4.6).

While stimulation in the mucosal LIF was observed for all age group in RT-TE harbors different bacteria in their tonsils (Table 4.7).

Comparison between systemic and mucosal LIF in RT-TE was observed using paired t-test. As shown in table (4.8) mucosal LIF shows an increased stimulation in comparison with systemic LIF for all RT-patient, but mucosal LIF in the tonsils harbors mixed bacteria show a significant increase.

Table 4.4: Systemic LIF in RT-TE harbors *S.aureus*, *S.pyogenes*, or mixed bacteria in their tonsils.

Age(years)	Systemic LIF in RT-TE			P-value
	<i>S.aureus</i>	<i>S.pyogenes</i>	Mixed bacteria	
5-9	0.7605± 0.0855	0.8653 ± 0.1765	0.8699± 0.1099	0.751
10-14	0.9216± 0.0736	1.0784 ± 0.2612	0.8222± 0.0874	0.402
15-19	0.9970± 0.0611	0.8621 ± 0.0949	1.1428± 0.3200	0.454
20-30	1.1110± 0.1110	1.0143± 0.0340	1.2448± 0.1954	0.597

P<0.05 is Significant.

Table 4.5: Systemic LIF in control groups against *S.aureus*, *S.pyogenes*, and mixed bacterial antigens.

Age(years)	Systemic LIF in control groups			P-value
	<i>S.aureus</i>	<i>S.pyogenes</i>	Mixed bacteria	
5-9	1.0800± 0.0583	0.9600 ± 0.0400	1.0000± 0.0447	0.242
10-14	1.0000± 0.0707	0.9400 ± 0.0244	1.0600± 0.0244	0.217
15-19	1.1600± 0.0509	1.0600 ± 0.0748	1.1000± 0.0774	0.599
20-30	1.1400± 0.0812	1.0000 ± 0.0447	0.9800± 0.0374	0.145

P<0.05 is Significant.

Table 4.6: Comparison of systemic LIF between RT-TE harbors *S.aureus*, *S.pyogenes*, or mixed bacteria in their tonsils and control groups.

RT-TE harbors	Age (years)	Systemic LIF %		P-value
		Patients	Controls	
<i>S.aureus</i>				
	5-9	0.7605 ± 0.0855	1.0800 ± 0.0583	0.059
	10-14	0.9216 ± 0.0736	1.0000 ± 0.0707	0.488
	15-19	0.9970 ± 0.0611	1.1600 ± 0.0509	0.075
	20-30	1.1110 ± 0.1110	1.1400 ± 0.0812	0.837
<i>S.pyogenes</i>				
	5-9	0.8653 ± 0.1765	0.9600 ± 0.0400	0.729
	10-14	1.0784 ± 0.2612	0.9400 ± 0.0244	0.625
	15-19	0.8621 ± 0.0949	1.0600 ± 0.0748	0.171
	20-30	1.0143 ± 0.0340	1.0000 ± 0.0447	0.801
Mixed bacteria				
	5-9	0.8699 ± 0.1099	1.0000 ± 0.0447	0.288
	10-14	0.8222 ± 0.0874	1.0667 ± 0.0210	0.020*
	15-19	1.1428 ± 0.3200	1.1000 ± 0.0774	0.904
	20-30	1.2448 ± 0.1954	0.9800 ± 0.0374	0.366

P<0.05 is Significant.

Table 4.7. : Mucosal LIF in RT-TE harbors *S.aureus*, *S.pyogenes*, or mixed bacteria in their tonsils.

Age(years)	Mucosal LIF in RT-TE			P-value
	<i>S.aureus</i>	<i>S.pyogenes</i>	Mixed bacteria	
5-9	1.0385 ± 0.1536	1.0390 ± 0.1033	1.3497 ± 0.1219	0.175
10-14	1.3743 ± 0.1189	1.5900 ± 0.1089	1.3053 ± 0.1491	0.442
15-19	1.3664 ± 0.2671	1.0335 ± 0.1684	1.5705 ± 0.2880	0.259
20-30	1.5167 ± 0.3876	1.0354 ± 0.1855	1.5404 ± 0.3300	0.468

P<0.05 is Significant

Table 4.8: Comparison between systemic and mucosal LIF in RT-TE harbors *S.aureus*, *S.pyogenes*, or mixed bacteria in their tonsils.

RT-TE harbors	Age (years)	LIF %		P-value
		Systemic	Mucosal	
S.aureus				
	5-9	0.7605 ± 0.0855	1.0385 ± 0.1536	0.122
	10-14	0.9216 ± 0.7361	1.3743 ± 0.1189	0.012*
	15-19	0.9970 ± 0.0611	1.3664 ± 0.2671	0.243
	20-30	1.1110 ± 0.1110	1.5167 ± 0.3876	0.571
S.pyogenes				
	5-9	0.8653 ± 0.1765	1.0390 ± 0.1033	0.385
	10-14	1.0784 ± 0.2612	1.5900 ± 0.1089	0.038*
	15-19	0.8621 ± 0.0949	1.0335 ± 0.1684	0.191
	20-30	1.0143 ± 0.0340	1.0354 ± 0.1855	0.911
Mixed bacteria				
	5-9	0.8699 ± 0.1099	1.3497 ± 0.1219	0.019*
	10-14	0.8222 ± 0.0874	1.3053 ± 0.1491	0.003*
	15-19	1.1428 ± 0.3200	1.5705 ± 0.2880	0.023
	20-30	1.2448 ± 0.1954	1.5404 ± 0.3300	0.466

* P<0.05 is Significant.

4.4. Identification of T cells (E-rosette):

E-rosette-forming T lymphocytes (Fig.4.2) were determined in peripheral blood of 103 RT-TE and 40 age matched controls.

Systemic E-rosette-forming T lymphocytes was significantly elevated ($p<0.05$) in RT-TE as compared with age matched controls.

The percent of E-RF cells in RT-TE harbors *S.pyogenes* show a slight decrease percentage in compare with those harbors *S.aureus* and mixed bacteria (Table 4.9).

At mucosal level, RT-TE harbors mixed bacteria show a marked decrease ($p<0.05$) in E-RF cells in the first age group in compare to those carry *S.aureus* & *S.pyogenes* in their tonsils.

10-14 years old patient show no significant differences in their mucosal E-RF cell. While adolescent and adult patient harbors *S.aureus* in their tonsils show a significant decrease ($p<0.01$) in E-RFC (Table 4.10).

As shown in table (4.11), systemic and mucosal E-RFC show significant differences ($P<0.05$) nearly in all age groups for RT-patient harbors *S.aureus*, *S.pyogenes*, and mixed bacteria in their tonsils. While those patients harbors *S.pyogenes* in their tonsils systemic and mucosal E-RFC did not change significantly.

As illustrated in table (4.12), age had no effect on the systemic E-RFC while mucosal E-RFC was more influenced by the age specially those patients harbors *S.aureus* and mixed bacteria in their tonsils (Table 4.13).

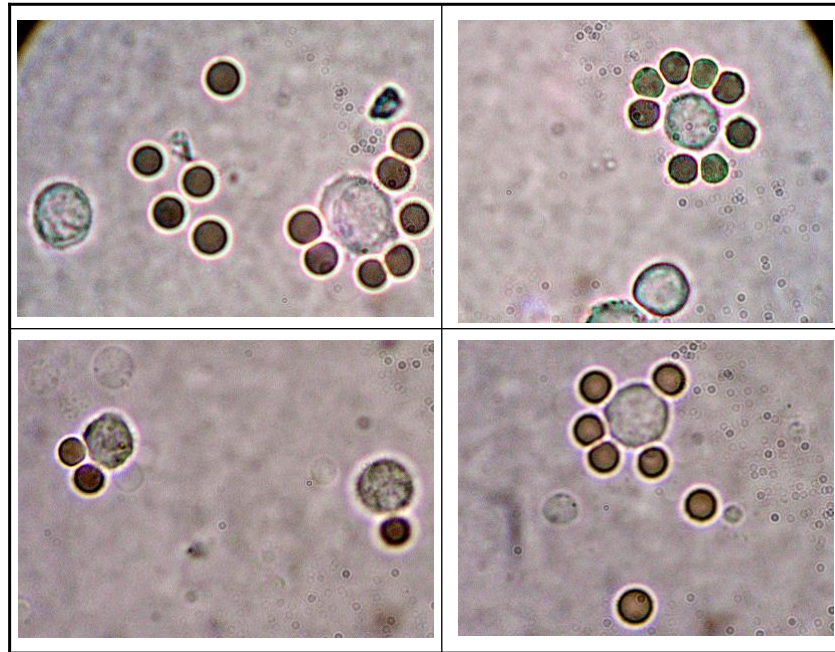


Fig.4.2: E-rosette-forming T lymphocyte. Lymphocyte binned with more than 3 sRBC considered positive cell (1000x).

Table 4.9: Systemic E-rosette in RT-TE harbors *S.aureus*, *S.pyogenes*, or mixed bacteria in their tonsils.

Age (years)	Systemic E-rosette in RT-TE %				P-value
	<i>S.aureus</i>	<i>S.pyogenes</i>	Mixed bacteria	Control	
5-9	36.955 ± 2.8158 ^b	37.977 ± 2.9318 ^b	32.601 ± 3.2764 ^b	14.375 ± 0.6952 ^a	0.000***
10-14	28.347 ± 4.6468 ^{ab}	22.924 ± 3.9460 ^{ab}	31.295 ± 2.1836 ^b	13.913 ± 1.6288 ^a	0.001**
15-19	37.261 ± 4.5595 ^b	27.666 ± 6.3716 ^{ab}	30.463 ± 6.1491 ^b	14.281 ± 1.3431 ^a	0.010*
20-30	41.423 ± 7.3660 ^b	28.663 ± 7.1684 ^{ab}	37.579 ± 5.8959 ^b	16.284 ± 1.0343 ^a	0.014*

*P<0.05 **P<0.01 ***P<0.001

Same alphabetic letters mean not significant.

Table 4.10: Mucosal E-rosette in RT-TE harbors *S.aureus*, *S.pyogenes*, or mixed bacteria in their tonsils.

Age(years)	Mucosal E-rosette in RT-TE %			P-value
	<i>S.aureus</i>	<i>S.pyogenes</i>	Mixed bacteria	
5-9	23.138 ± 1.1762 ^b	22.052 ± 2.2453 ^{ab}	17.224 ± 1.9106 ^a	0.047*
10-14	25.778 ± 4.6256 ^b	15.524 ± 2.0379 ^a	21.856 ± 1.5062 ^{ab}	0.134
15-19	13.807 ± 1.2454 ^a	18.601 ± 2.9909 ^{ab}	27.871 ± 4.0241 ^b	0.035*
20-30	12.819 ± 0.0607 ^a	22.005 ± 2.3997 ^b	14.523 ± 0.9196 ^a	0.004***

*P<0.05 **P<0.01

Table 4.11: Comparison between systemic and mucosal E-rosette in RT-TE harbors *S.aureus*, *S.pyogenes*, or mixed bacteria in their tonsils.

RT-TE harbors	Age (year)	E-rosette %		P-value
		Systemic	Mucosal	
S.aureus				
	5-9	36.955 ± 2.8158	23.138 ± 1.1762	0.000*
	10-14	28.347 ± 4.6468	25.778 ± 4.6256	0.554
	15-19	37.261 ± 4.5595	13.807 ± 1.2454	0.004*
	20-30	41.423 ± 7.3660	12.819 ± 0.0607	0.061
Total	5-30	35.270 ± 2.1098	21.373 ± 1.5134	0.000*
S.pyogenes				
	5-9	37.977 ± 2.9318	22.052 ± 2.2453	0.002*
	10-14	22.924 ± 3.9460	15.524 ± 2.0379	0.183
	15-19	27.666 ± 6.3716	18.601± 2.9909	0.227
	20-30	28.663 ± 7.1684	22.005 ± 2.3997	0.406
Total	5-30	30.785 ± 2.6792	20.098 ± 1.2994	0.001*
Mixed bacteria				
	5-9	32.601 ± 3.2764	17.224 ± 1.9106	0.001*
	10-14	31.295 ± 2.1836	21.856 ± 1.5062	0.019*
	15-19	30.463 ± 6.1491	27.871 ± 4.0241	0.815
	20-30	37.579 ± 5.8959	14.523 ± 0.9196	0.003*
Total	5-30	33.273 ± 2.0611	18.937 ± 1.1132	0.000*

*P<0.05 is Significant.

Table 4.12: Effect of age on systemic E-rosette forming cell in RT-TE harbors *S.aureus*, *S.pyogenes*, and mixed bacteria in their tonsils.

RT-TE harbors	Systemic E-rosette in RT-TE %				P-value
	5-9	10-14	15-19	20-30	
<i>S.aureus</i>	36.955 ± 2.8158	28.347 ± 4.6468	28.347 ± 4.6468	41.423 ± 7.3660	0.275
<i>S.pyogene</i>	37.977 ± 2.9318	22.924 ± 3.9460	27.666 ± 6.3716	28.663 ± 7.1684	0.221
Mixed bacteria	32.601 ± 3.2764	31.295 ± 2.1836	30.463 ± 6.1491	37.579 ± 5.8959	0.682
Control	14.375 ± 0.6952	13.913 ± 1.6288	14.281 ± 1.3431	16.284 ± 1.0343	0.526

* P<0.05 is Significant.

Table 4.13: Effect of age on mucosal E-rosette forming cell in RT-TE harbors *S.aureus*, *S.pyogenes*, and mixed bacteria in their tonsils

RT-TE harbors	Mucosal E-rosette in RT-TE %				P-value
	5-9 yr.	10-14 yr.	15-19 yr.	20-30 yr.	
<i>S.aureus</i>	23.138 ± 1.1762 ^b	25.778 ± 4.6256 ^b	13.807 ± 1.2454 ^a	12.819 ± 0.0607 ^a	0.014*
<i>S.pyogenes</i>	22.052 ± 2.2453	15.524 ± 2.0379	18.601 ± 2.9909	22.005 ± 2.3997	0.315
Mixed bacteria	17.224 ± 1.9106 ^{ab}	21.856 ± 1.506 ^{ab}	27.871 ± 4.0241 ^b	14.523 ± 0.9196 ^a	0.002***

* P<0.05 **P<0.01 ***P<0.001

4.5. Specific Antibody to cell free culture antigen:

Specific antibodies for CFC antigen were determined for *S.aureus*, *S.pyogenes*, and mixed bacteria. For mixed bacteria amount of whole cell bacterial antigen from different bacterial species were titrated against patient sera and TIg in way similar to multiplicity test described by Kwapinski (1972) who had mentioned multiplicity neutralization test in which employs mixture of 2 different Ag in constant amount against varying dilution of antiserum. Titers were noted ranging between 20-80 for systemic, while they were 40-320 for mucosal.

The titers of specific antibody and their frequency were determined in both serum and mucosal immunoglobulin extraction (TIGs) as shown in table below.

Table 4.14: Profile of specific antibody to CFC antigen for *S.aureus*, *S.pyogenes*, and mixed bacteria in both Systemic and Mucosal immunity.

Bacteria									Control	
S.aureus			S.pyogenes			Mixed bacteria				
Systemic	Mucosal	F.	Systemic	Mucosal	F.	Systemic	Mucosal	F.	Systemic	F
20	40	2	20	80	3	20	40	8	10	2
20	80	5	20	160	5	20	80	7	20	5
40	80	12	40	80	16	40	80	16	40	3
40	160	13	40	160	5	40	160	5		
			80	320	2	40	320	2		
						80	320	2		

F. (Frequency)

For the patient harbors *S.aureus*, *S.pyogenes*, and Mixed bacteria in their tonsils the most common titer was 40 in their serum, While the most common titer in their tonsils was 160 in those harbors *S.aureus* and 80 for those harbors *S.pyogenes* and mixed bacteria.

On the other hand, the most common titer was 20 in control group.

4.6. Cytokine detection:

Sample results are calculated by interpolation from the calibration curve that is performed in the same assay with samples.

Calibration curve was drawing by plotting cytokines concentration of the calibrators on the horizontal axis and corresponding absorbance in the vertical axis, then by using curve fit equation (quadratic mode) from SPSS package and Excel programs we obtained the concentration of each samples (Table 4.15, 16, 17, 18).

Systemic IL-6 and IFN-gamma show no significant differences among RT-TE harbors different bacteria in their tonsils, on the other hand, IL-10 show significant increase ($P < 0.001$) in RT- patient harbors *S.aureus* (88.820 ± 6.750 pg/ml), *S.pyogenes* (99.780 ± 5.640 pg/ml), and mixed bacteria (92.083 ± 6.214 pg/ml) in their tonsils in compare to control groups (59.391 ± 8.044 pg/ml) as shown in table 4.19.

No significant difference in mucosal IL-6 was observed among RT-TE while mucosal IL-10 show a significant increase ($P < 0.05$) in RT-TE harbors *S.pyogenes* in their tonsils (196.810 ± 16.071 pg/ml) in compare to those harbor *S.aureus* or mixed bacteria (137.647 ± 18.512 pg/ml and 153.753 ± 6.017 pg/ml) (table 4.20).

On the other hand, IFN- γ show a significant decrease ($P < 0.001$) in RT-patient harbors mixed bacteria in their tonsils (1.198 ± 0.100 IU/ml) in compare to those harbors *S.pyogenes* (2.286 ± 0.134 IU/ml) or *S.aureus* (2.071 ± 0.183 IU/ml).

Statistical analysis using paired t-test revealed that local cytokine concentration was increased significantly ($P < 0.05$) for IL-6, IL-10 and IFN-gamma in compare to systemic cytokine concentration (table 4.21).

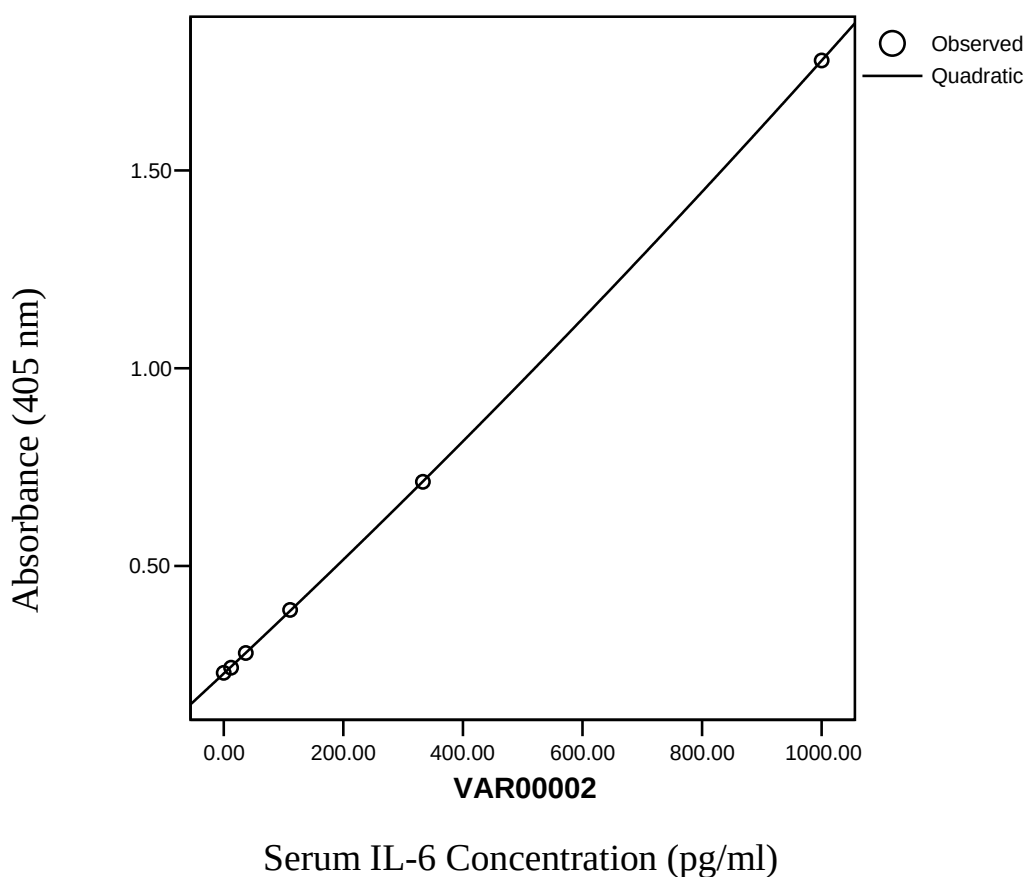
Table 4.15: Statistical feature for standard curve of serum IL-6 (Quadratic mode).

Calibrator IL-6 (pg/ml) (X)	1000	333	111	37	12.3	0
Absorbance (mOD) (Y)	1.778	0.713	0.389	0.280	0.243	0.230

Independent: VAR00002

Dependent	Mth	Rsqr	d.f.	F	Sigf	A	B	C
VAR00001	QUA	1.000	3	4622.61	.000	.2283	.0014	1.4E-07

VAR00001



$$Y = A + BX + CX^2$$

$$X = (-B + \text{SQRT}(B^2 - 4C(A - y))) / (2C)$$

$$X = (-0.0014 + \text{SQRT}(0.0014^2 - 4 \times 1.8 \times 1/10000000(0.2378 - Y))) / (2 \times 1.8 \times 1/10000000)$$

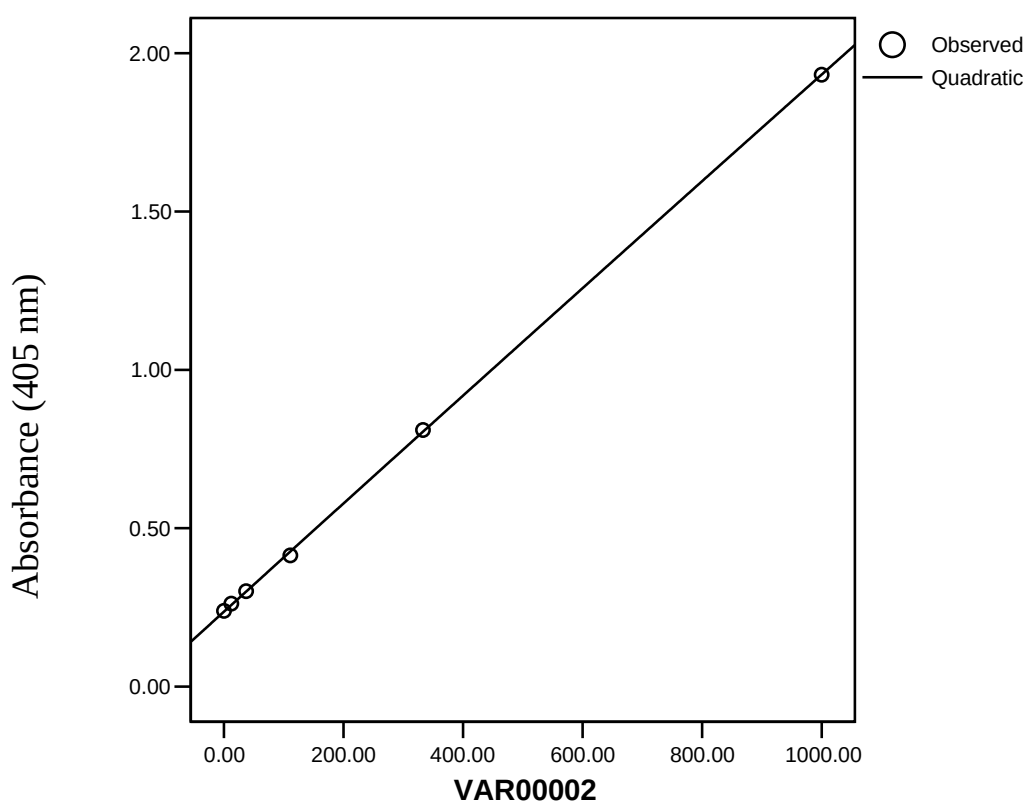
Table 4.16 : Statistical feature for standard curve of tissue IL-6 (Quadratic mode).

Calibrator IL-6 (pg/ml) (X)	1000	333	111	37	12.3	0
Absorbance (mOD) (Y)	1.932	0.810	0.414	0.301	0.262	0.239

Independent: VAR00002

Dependent	Mth	Rsq	d.f.	F	Sigf	A	B	C
VAR00001	QUA	1.000	3	15726.0	.000	.2366	.0017	-2.E-08

VAR00001



Tissues IL-6 Concentration (pg/ml)

$$Y = A + BX + CX^2$$

$$X = (-B + \text{SQRT}(B^2 - 4C(A - y))) / (2C)$$

$$X = (-0.0017 + \text{SQRT}(0.0017^2 - 4 \times (-2 \times 1/100000000)(0.2366 - Y))) / (2 \times -2 \times 1/100000000)$$

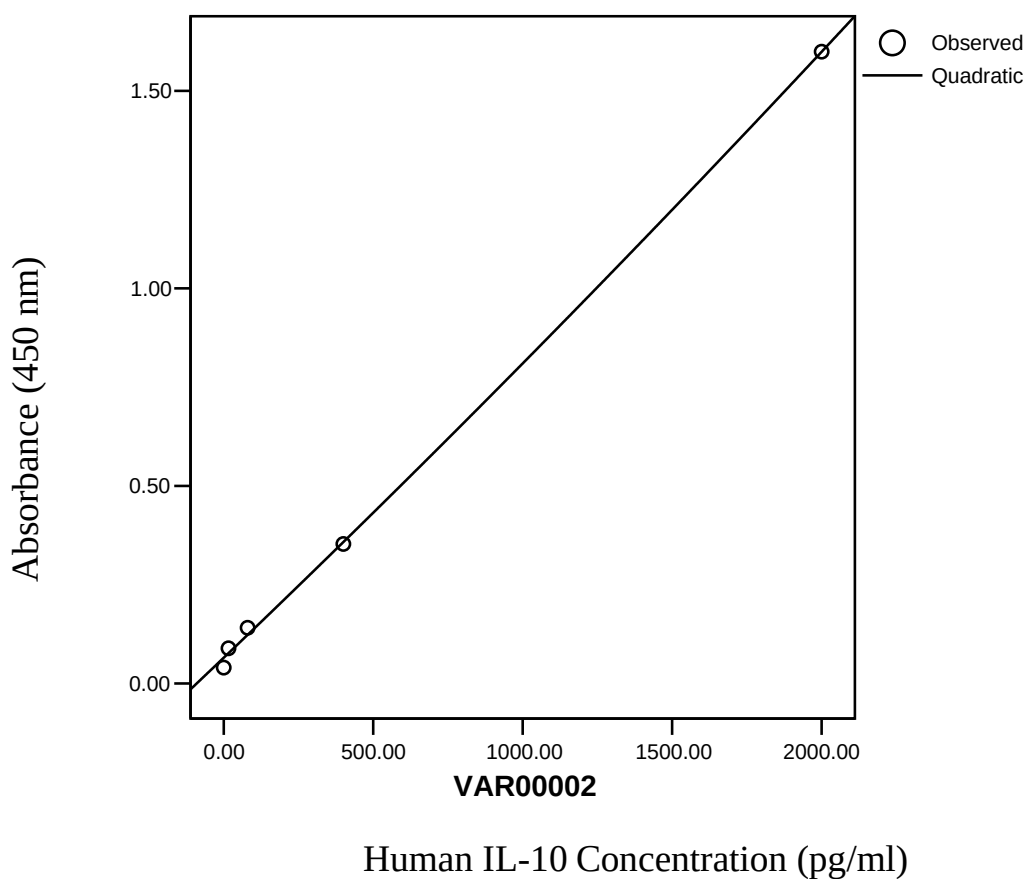
Table 4.17 : Statistical feature for standard curve of IL-10(Quadratic mode).

Calibrator IL-10 (pg/ml) (X)	2000	400	80	16	0
Absorbance (mOD) (Y)	1.599	0.353	0.141	0.089	0.040

Independent: VAR00002

Dependent	Mth	Rsq	d.f.	F	Sigf	A	B	C
VAR00001	QUA	.999	2	1529.30	.001	.0653	.0007	2.2E-08

VAR00001



$$Y = A + BX + CX^2$$

$$X = (-B + \text{SQRT}(B^2 - 4C(A - y))) / (2C)$$

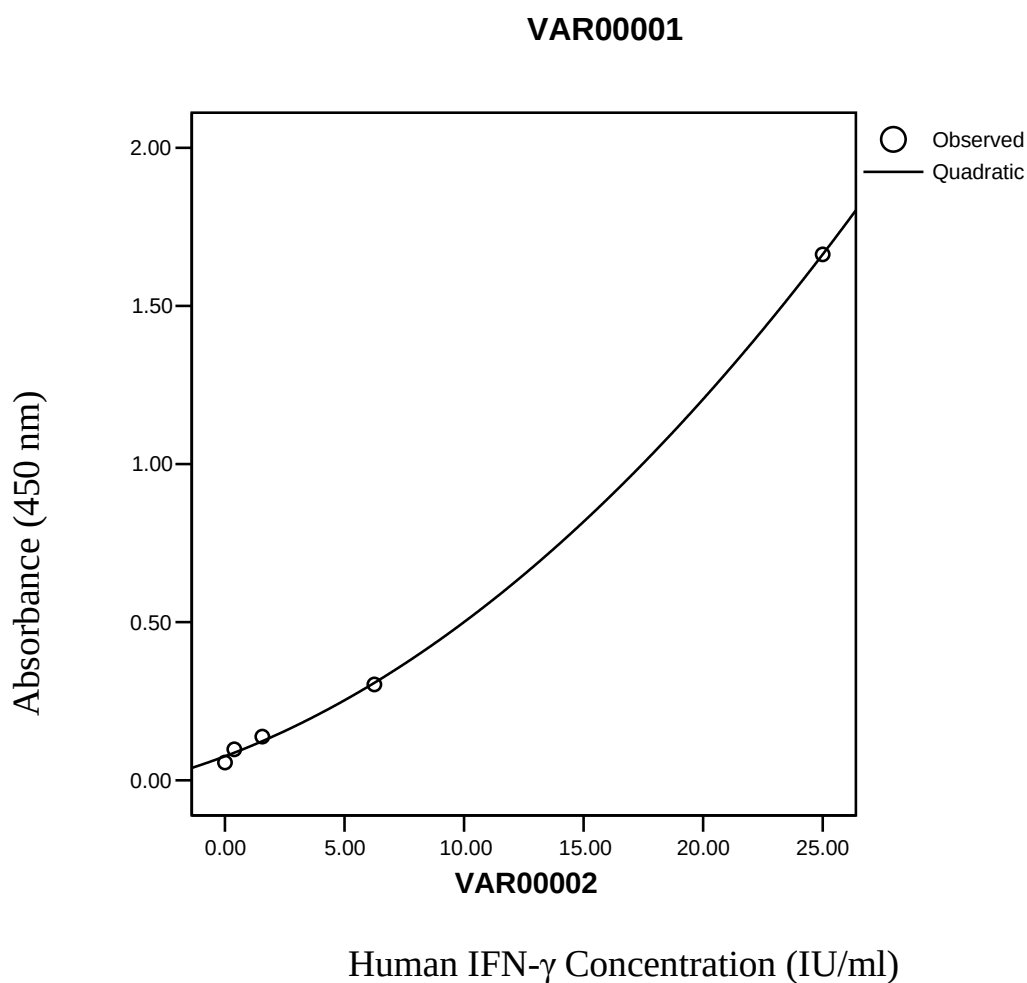
$$X = (-0.0007 + \text{SQRT}(0.0007^2 - 4 \times 2.2 \times 1/100000000 (0.653 - Y))) / (2 \times 2.2 \times 1/100000000)$$

Table 4.18 :Statistical feature for standard curve of IFN- γ (Quadratic mode).

Calibrator IFN- γ (IU/ml) (X)	25	6.25	1.56	0.39	0
Absorbance (mOD) (Y)	1.663	0.303	0.138	0.098	0.056

Independent: VAR00002

Dependent	Mth	Rsqr	d.f.	F	Sigf	A	B	C
VAR00001	QUA	1.000	2	2502.24	.000	.0760	.0284	.0014



$$Y = A + BX + CX^2$$

$$X = (-B + \text{SQRT}(B^2 - 4C(A - y))) / (2C)$$

$$X = (-0.0284 + \text{SQRT}(0.0284^2 - 4 \times 0.0014(0.0760 - Y))) / (2 \times 0.0014)$$

Table 4.19: Concentration of Systemic Cytokines in RT-TE harbors *S.aureus*, *S.pyogenes*, Mixed bacteria in their tonsils.

Systemic Cytokine	Bacteria				P-value
	<i>S.aureus</i>	<i>S.pyogenes</i>	Mixed bacteria	Control	
IL-6 (pg/ml)	8.3464± 2.0580	14.557 ± 2.2073	10.328± 1.6666	9.0598± 2.1976	0.193
IL-10 (pg/ml)	88.820± 6.7505 ^{ab}	99.780 ± 5.6402 ^b	92.083± 6.2148 ^{ab}	59.391± 8.0449 ^a	0.001***
IFN- γ (IU/ml)	0.3551± 0.0807	0.3677 ± 0.0552	0.4424± 0.1386	0.3544± 0.1145	0.908

***P<0.001

Table 4.20: Concentration of Mucosal Cytokines in RT-TE harbors *S.aureus*, *S.pyogenes*, Mixed bacteria in their tonsils.

Mucosal Cytokine	Bacteria			P-value
	<i>S.aureus</i>	<i>S.pyogenes</i>	Mixed bacteria	
IL-6 (pg/ml)	85.175 ± 17.190	63.824 ± 13.127	53.866 ± 5.3954	0.232
IL-10 (pg/ml)	137.64 ± 18.512 ^a	196.81 ± 16.071 ^b	153.75 ± 6.0175 ^a	0.022*
IFN-γ (IU/ml)	2.0717 ± 0.1837 ^b	2.2861 ± 0.1344 ^b	1.1988 ± 0.1003 ^a	0.000***

*P<0.05 *** P<0.001

Table 4.21: Comparison between Systemic and Mucosal Cytokine in RT-TE harbors *S.aureus*, *S.pyogenes*, or Mixed bacteria in their tonsils.

Type of Cytokine	RT-TE harbors	Cytokine Concentration		P-value
		Systemic	Mucosal	
IL-6 (pg/ml)				
	<i>S.aureus</i>	8.3464±2.0580	85.175±17.190	0.001*
	<i>S.pyogenes</i>	14.557±2.2073	63.824±13.127	0.012*
	Mixed bacteria	10.328±1.6666	53.866±5.3954	0.000*
IL-10 (pg/ml)				
	<i>S.aureus</i>	88.820±6.7505	137.64±18.512	0.03*
	<i>S.pyogenes</i>	99.780±5.6402	196.81±16.071	0.000*
	Mixed bacteria	92.083±6.2148	153.75±6.0179	0.000*
IFN-γ (IU/ml)				
	<i>S.aureus</i>	0.3551±0.0807	2.0717±0.1837	0.000*
	<i>S.pyogenes</i>	0.3677±0.0552	2.2861±0.1344	0.000*
	Mixed bacteria	0.4424±0.1386	1.1988±0.1003	0.006*

*P<0.05 is Significant.

4.7. Light Microscopy:

Several histological changes have been observed in all compartment of the tonsils including tonsillar crypt and epithelium, extra follicular area, and follicles of the tonsils harbors *S.aureus*, *S.pyogenes*, and mixed bacteria.

4.7.1. Tonsillar crypts and epithelium:

The palatine tonsil was covered with stratified squamous epithelium of 8.4µm thickness (Fig.4.3). Approximately 11.2µm Thickness exhibiting a compact pattern with occasional infiltration lymphocytes was shown in tonsils harbors *S.aureus* (Fig.4.4.A).

Tonsils harbors *S.pyogenes* shown 15µm thicknesses with mild to sever distruction in some patches of the epithelium surface (Fig.4.4.B). While the tonsils harbors mixed bacteria show 15.6 µm thicknesses with mild separation of the surface epithelial cell (Fig.4.4.C).

Statistical analysis of the thickness of epithelium surface show a significant differences ($p < 0.001$) between the thickness of epithelium in normal tonsils and tonsils harbor *S.aureus* and thickness of epithelium in tonsils harbor *S.pyogenes* or mixed bacteria (Table 4.22).

Table 4.22: Thickness of epithelium in RT-TE harbors *S.aureus* or *S.pyogenes* or mixed bacteria in their tonsils.

	RT-TE Harbors			Control	P-value
	<i>S.aureus</i>	<i>S.pyogenes</i>	Mixed bacteria		
Thickness of epithelium (µm)	11.207±0.3309 ^B	15.053±0.6386 ^c	15.608±0.5015 ^c	8.4571±0.2201 ^a	0.000***

***P < 0.001

Same alphabetic letters mean not significant.

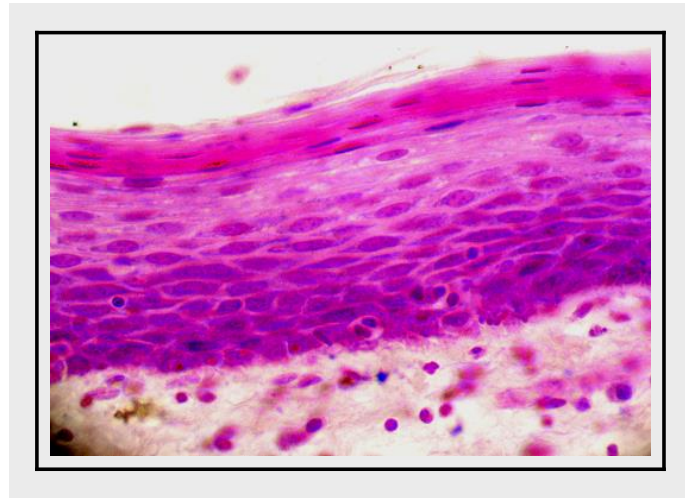


Fig.4.3: Epithelial layer of normal palatine tonsils. H&E, 400x. (Gen. Biol. Sup.House, Chicago, U.S.A).

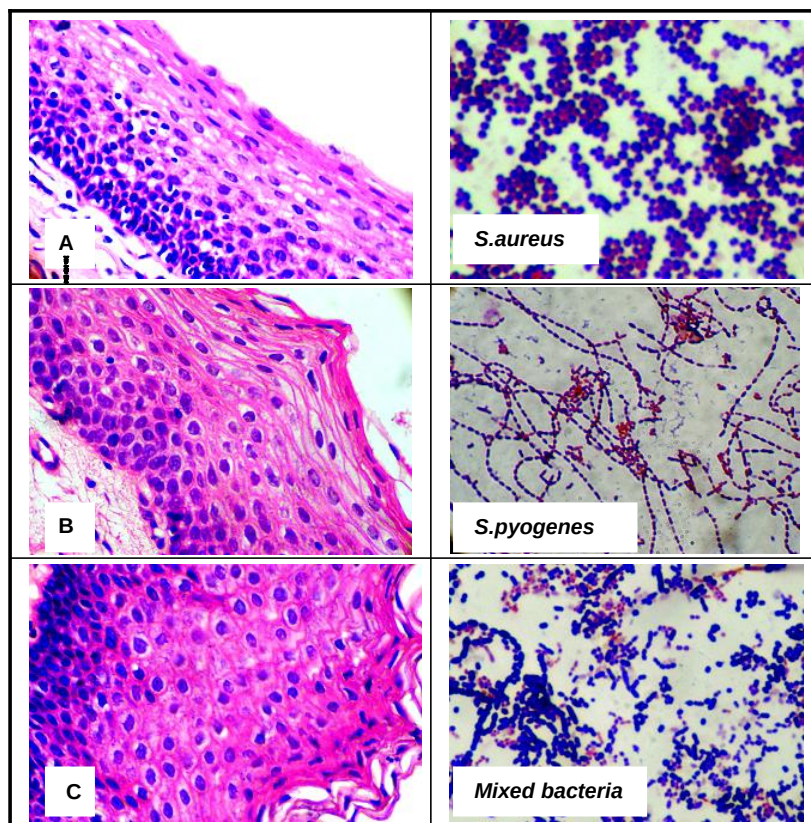


Fig.4.4: Epithelial layer of chronically inflamed tonsils harbor *S.aureus* (A), *S.pyogenes* (B), and mixed bacteria (C).H&E, 400x.

This epithelium was supported by a layer of collagenous connective tissue, which carried a network of blood vessels and separated it from the underlying lymphoid tissue (Fig.4.5.A).

The epithelium lining the crypts is a highly modified from the stratified squamous epithelium that covers the oropharyngeal surface of the tonsils.

The crypt epithelium was usually infiltrated by lymphocytes showing a mixed feature called "simbiosi linfoepithelial " (the lymphoepithelial symbiosis) (Fig.4.5.B).

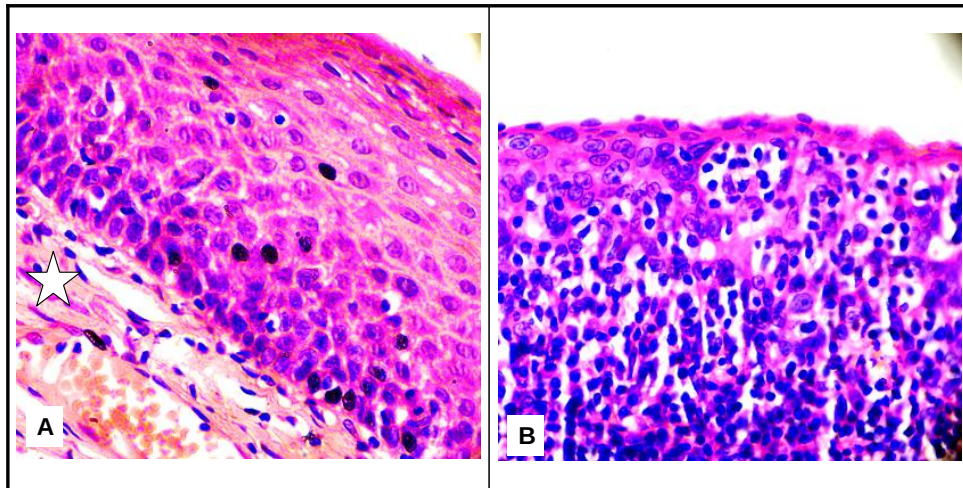


Fig.4.5: The human palatine tonsils (A) surface of the palatine tonsils covered with stratified squamous epithelium, underlined by a connective tissue band (five point stare)H&E, 400x (B) Reticulated crypt epithelium containing many lymphatic cells.H&E, 400x.

4.7.2. Infiltrating non epithelial cells:

The morphology of the epithelial cell was often altered where there were numerous non epithelial cells present. The following types of non epithelial cells were identified:

(1) Lymphocytes of various morphologies, found either singly or in groups (Fig.4.6 A, B). Clusters of lymphocytes were some times tightly packed in the epithelial cell network (Fig.4.7.B). No significant deference in lymphocyte infiltrating to surface epithelium were observed in tonsils harbor *S.aureus* or *S.pyogenes* or mixed bacteria as shown in table (4.23).

(2) Plasma cell, with abundant bluish cytoplasm and moderate size nucleus located at one pole of the cell, were identified in some patches of reticulated crypt epithelium as a predominant cell type (Fig.4.7.A), such pattern was not observed in the surface epithelium except for few filed (Fig.4.8).

No significant differences in plasma cell were observed among RT-patient harbors *S.aureus* or *S.pyogenes* or mixed bacteria within surface epithelium as in table (4.23).

(3) Polymorph nuclear leukocytes, mostly neutrophils, identified as a cluster of 5-40 cells in some area as in Figure (4.9 A, B) with single or no positive cells in other area. Neutrophil, mostly in the superficial layers of epithelium, were identified in tonsils harbor *S.aureus* and mixed bacteria, such picture were not identified in *S.pyogenes* which show a significant decrease ($p<0.05$) as in table (4.23).

(4) Erythrocytes either isolated or in small groups, lying between the epithelium was observed, especially in tonsils harbor *S.pyogenes* (Fig.4.10).

Table 4.23: Frequency of immune cells in the Surface epithelium, Follicles, and Extra follicular area of human Palatine tonsils in RT-TE harbors *S.aureus*, *S.pyogenes*, and Mixed bacteria in their tonsils.

Compartment of Tonsils	Immune Cells	RT-TE harbors			P-value
		<i>S.aureus</i>	<i>S.pyogenes</i>	Mixed bacteria	
Surface epithelium					
	Lymphocyte§	222.3±24.705	265.75 ± 58.277	162.36±24.724	0.177
	Polymorph nuclear leukocyte§	129.57±35.86 ^b	23.748 ± 7.2563 ^a	90.868±33.715 ^{ab}	0.033 [*]
	Plasma Cell§	4.8450±1.5015	3.7683 ± 1.3499	2.6917±1.1622	0.528
Follicles					
	Antigen Presenting Cell Ψ	185.80±8.5897	196.10 ± 9.6593	221.92±14.703	0.073
	Plasma cell Ψ	98.064±21.105	107.10 ± 11.880	144.50±18.910	0.153
Extra follicular area					
	Antigen Presenting Cell Ψ	135.36±15.834	175.48 ± 20.493	188.83±32.133	0.267
	Plasma Cell Ψ	101.93±20.371 ^b	46.464 ± 11.183 ^{ab}	27.112±8.4684 ^a	0.001 ^{**}

*P<0.05 **P<0.01 ***P<0.001

§ 12 randomly chosen ocular fields were counted in each sample.

Ψ 5 randomly chosen ocular fields were counted in each sample

‡ Number of cell was determined for 1mm².

⊖ Same alphabetic letter mean not significant.

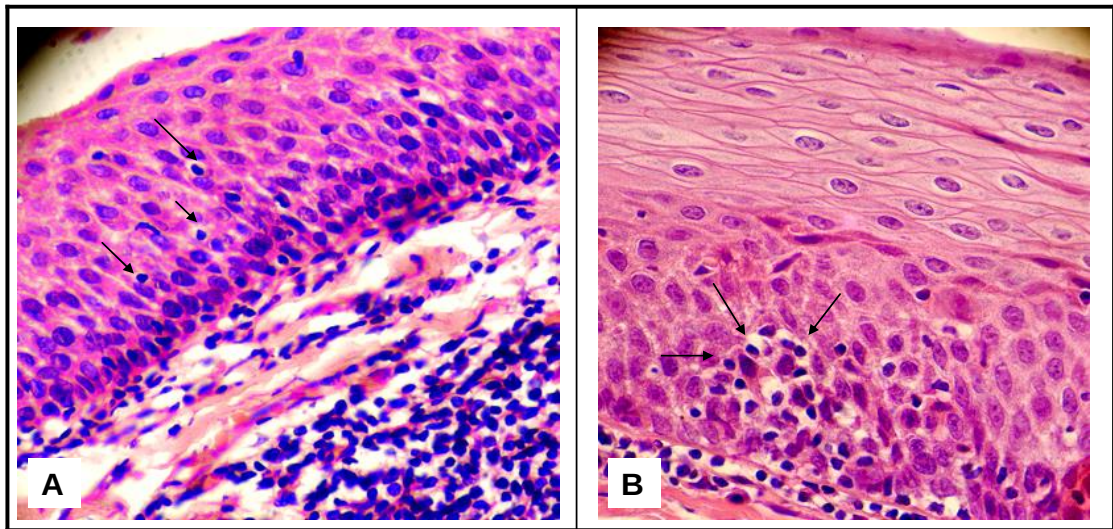


Fig.4.6: Surface epithelial contains (A) single lymphocyte (B) cluster of lymphocyte. H&E, 400x.

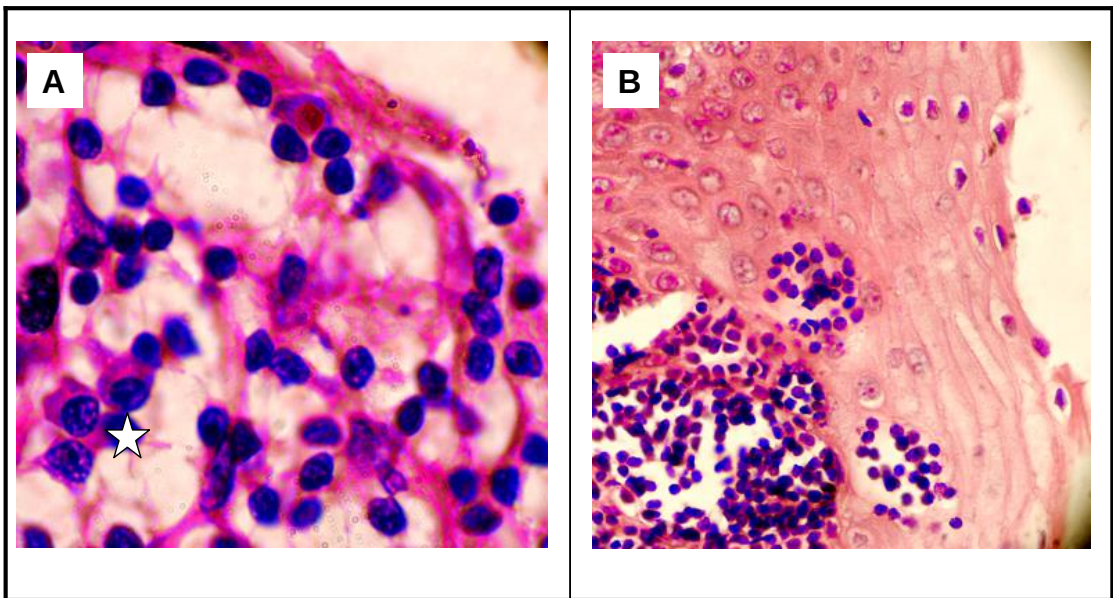


Fig.4.7: Reticulated crypt epithelium contains (A) 3 plasma cells (around stare), surrounded by lymphocytes and epithelial cell.(B) Cluster of lymphocyte tightly packed in epithelium surface H&E, 1000x.

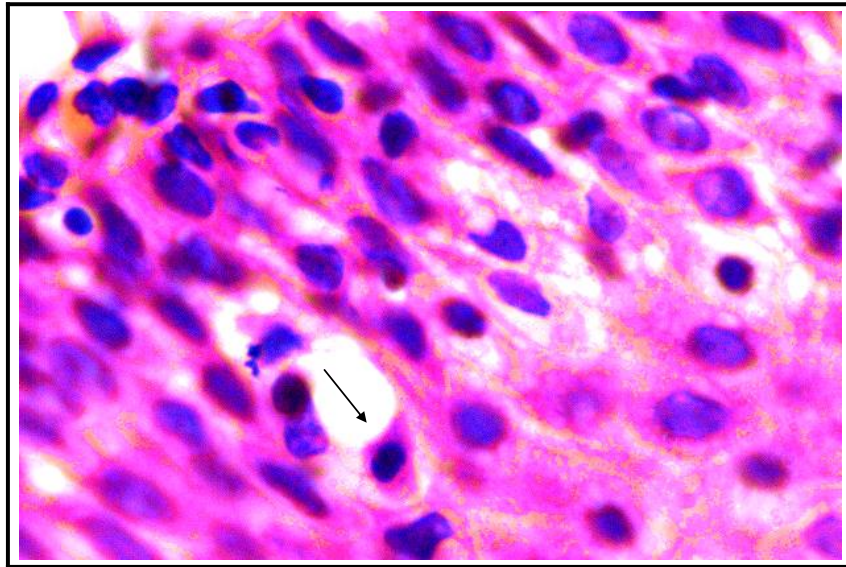


Fig.4.8: Plasma in the surface epithelium of tonsils harbors *S.aureus* bacteria. H&E, 1000x.

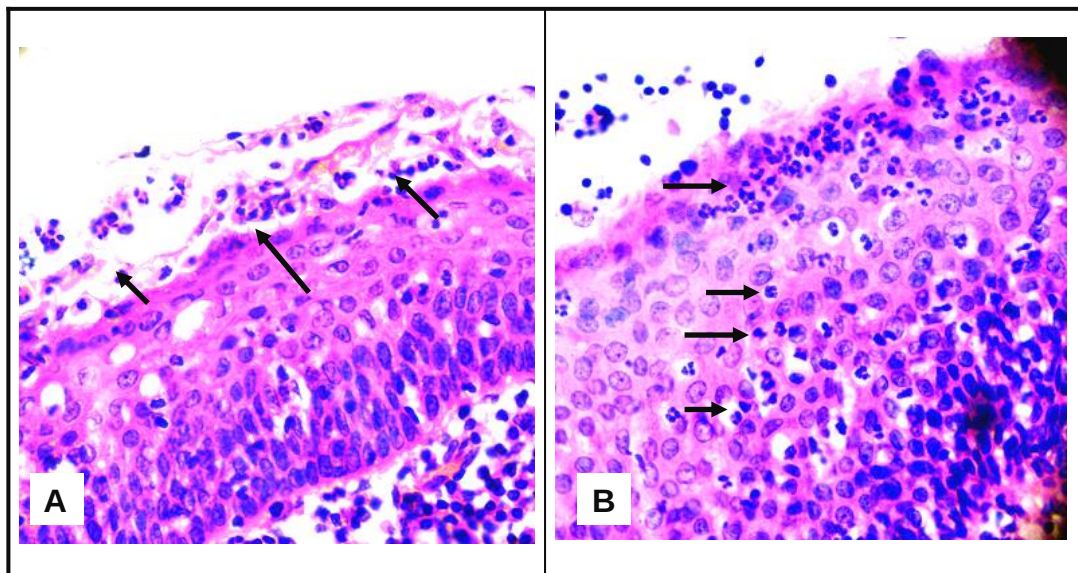


Fig.4.9: Section from a tonsil harbors *S.aureus*. (A) Cluster of neutrophils are seen in tonsillar surface. (B) Show neutrophils migration from small vessels in tonsillar parenchyma to the surface epithelium as a streak (short arrows). H&E, 400x.

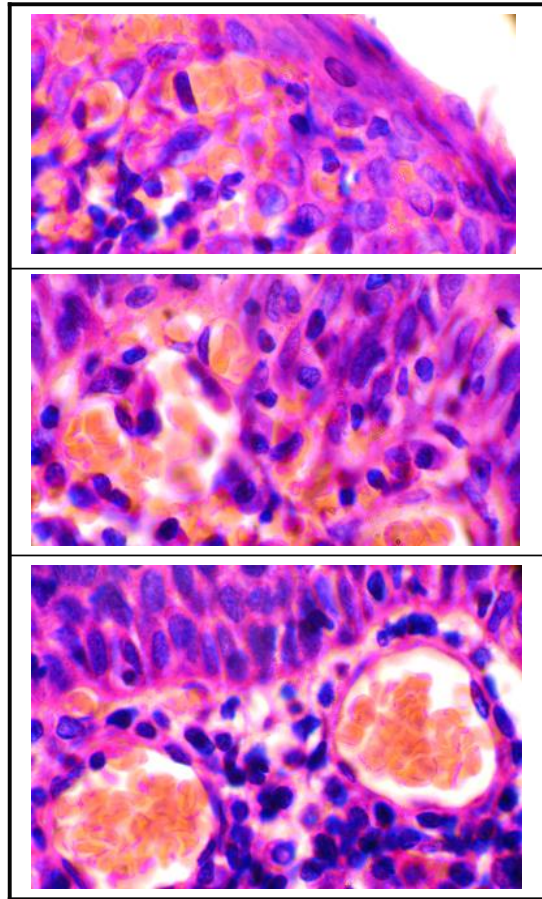


Fig.4.10: Distribution of blood in the epithelial layer of tonsils harbors *S.pyogenes*.H&E, 1000x.

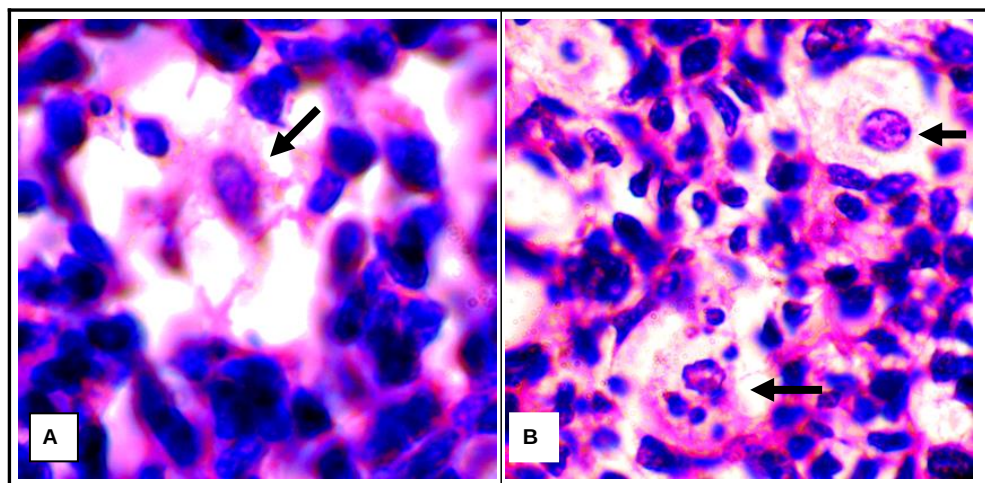


Fig.4.11: Antigen presenting cells in extra follicular region of palatine tonsils in patient with RT. (A) Dendritic cell with long cytoplasmic extension (B) Macrophage.H&E, 400x.

4.7.3. Extra follicular area:

Quantification of APC including both dendritic cell, with long cytoplasmic extension (Fig.4.11 A), and macrophages (Fig.4.11 B) was done in the extra follicular region. No significant differences in APCs were seen among the three groups within the extra follicular area while plasma cell increased significantly ($p < 0.001$) in tonsils harbors *S.aureus* in compare with tonsils harbor *S.pyogenes* or mixed bacteria (Table 4.23).

4.7.4. Follicular area:

Interestingly, there was a morphological difference between the follicles in tissue harbor *S.aureus* and mixed bacteria with those tissue harbors *S.pyogenes*. In the patient with RT harbor *S.pyogenes* the germinal centers had ill-defined borders (Fig.4.13 A, B). In contrast, tonsils of patient with RT harbor *S.aureus* and mixed bacteria had well defined germinal center borders and distinct morphological zones with germinal centers (Fig.4.14 A, B).

The sections of normal tonsils show physiological inflammation of tonsils with follicles show inactivation of cells inside and outside the follicles (Fig.4.12).

No significant differences were observed among the diameter of the follicle in the three groups. But a significant decrease ($p < 0.001$) in number of follicles was observed in the tonsils harbor *S.pyogenes* (Table 4.24).

Table 4.24: Number and diameter of follicles in RT-TE harbors *S.aureus* or *S.pyogenes* or mixed bacteria in their tonsils.

Parameter	RT-TE harbors			P-value
	<i>S.aureus</i> bacteria	<i>S.pyogenes</i>	mixed	
Number of Follicle ^α	12.588±1.2662 ^b	3.1765±0.8754 ^a	11.764±1.6349 ^b	0.000***
Diameter of follicle [§]	35.360± 2.5198	28.800±1.0392	34.080±2.7880	0.099

***P< 0.001

⊖Same alphabetic mean not significant, different letter mean significant.

αNumber of follicle was determined per field of 4x, which is equal to 19.642 mm².

§Diameter of follicles was determined in μm.

In the germinal centers there are many APC with clear cytoplasm which, against the background of a large population of deeply basophilic small lymphocytes.

No significant differences in number of APC in the follicle of the tonsils were observed for RT-TE harbors different bacteria (Table 4.23).

The follicular germinal centers are often associated with B-cell differentiation to memory cells and isotype-specific plasma cells (Fig.4.15 A, B).No significant differences in plasma cell were observed among the three groups as shown in table (4.23).

In general, *S.aureus* and mixed bacteria show more hypertrophied tonsils with less damage while tonsils invaded by *S.pyogenes* show more tissue damage, necrotized and atrophy tonsils as shown in figure (4.16 A, B).

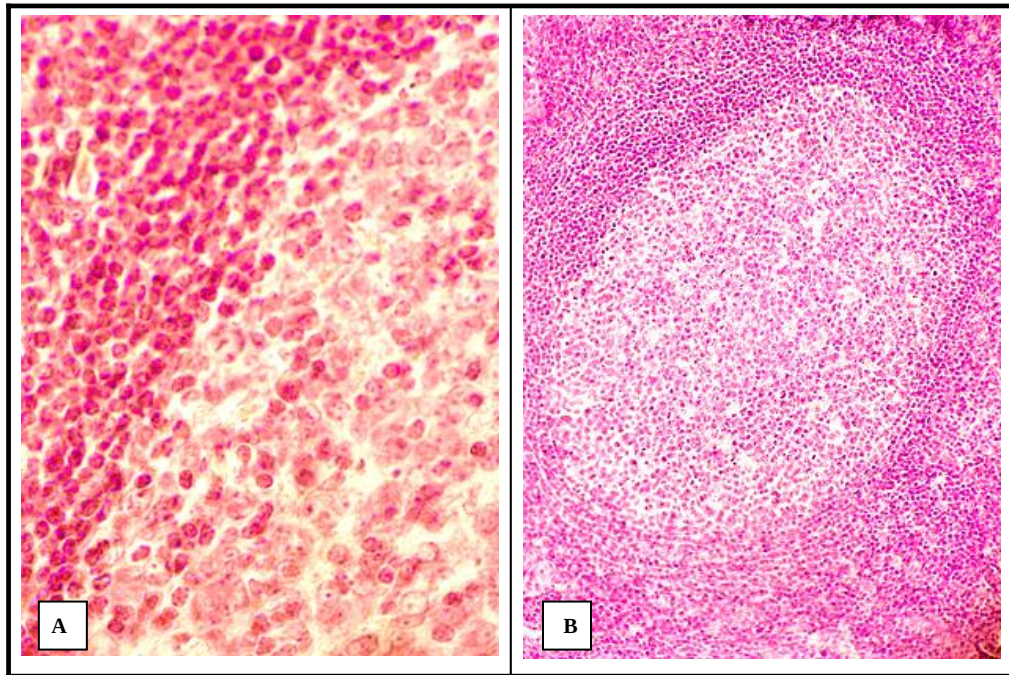


Fig.4.12: Germinal center in normal palatine tonsils show physiological inflammation. H&E, (A) 400x. (B) 100x (Gen.Biol.Sup.House, Chicago, U.S.A).

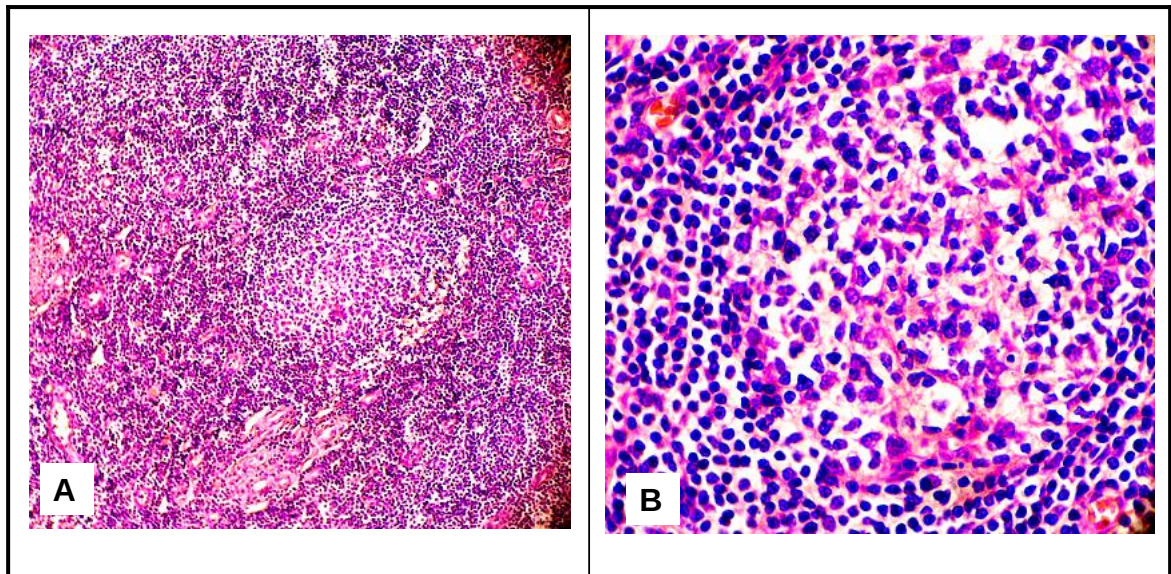


Fig.4.13: Germinal center in chronically inflamed tonsils harbor *S.pyogenes* (A) H&E, 100x. (B) Magnification of A.H&E, 400x.

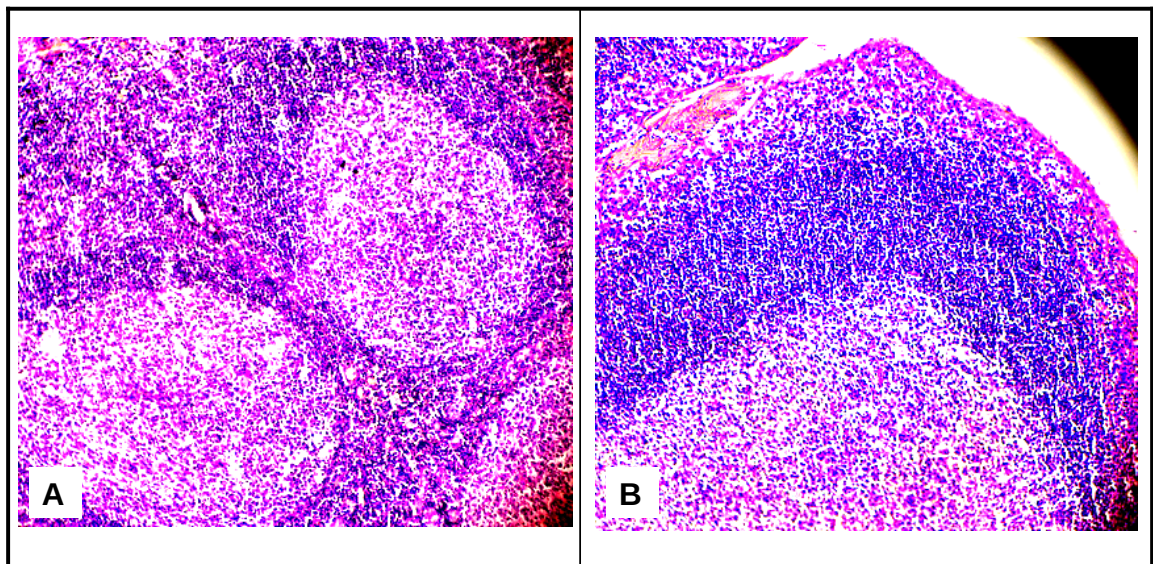


Fig.4.14: Germinal center in chronically inflamed tonsils harbor *S.aureus*.H&E, 100x (A). (B) Under magnification of 400x.H&E.

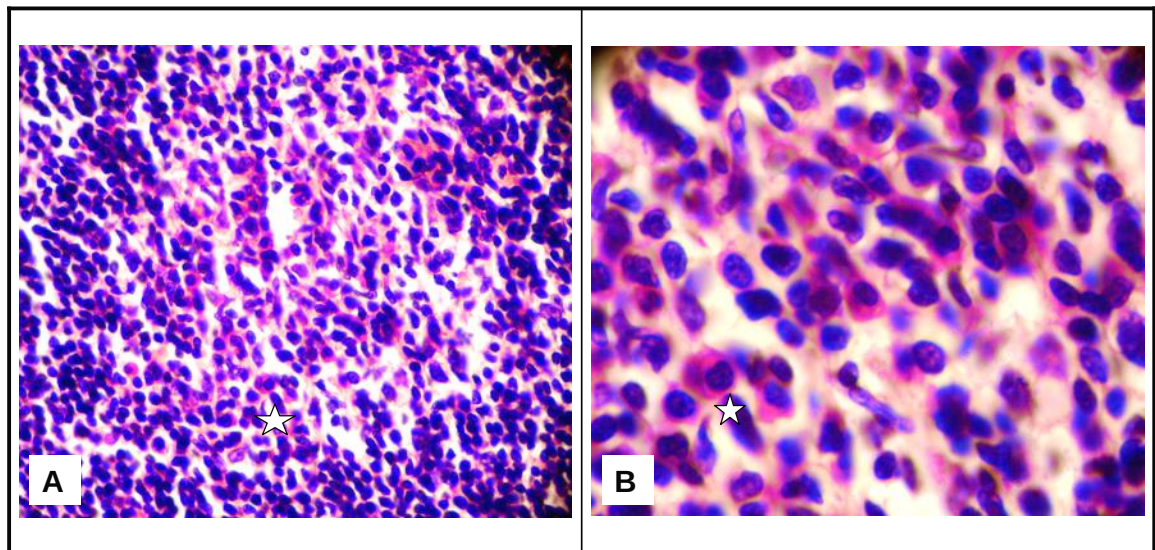


Fig.4.15: Plasma cell in the follicular area of tonsils harbors mixed bacteria (A).H&E, 400x. (B) Magnification of (A).1000x.

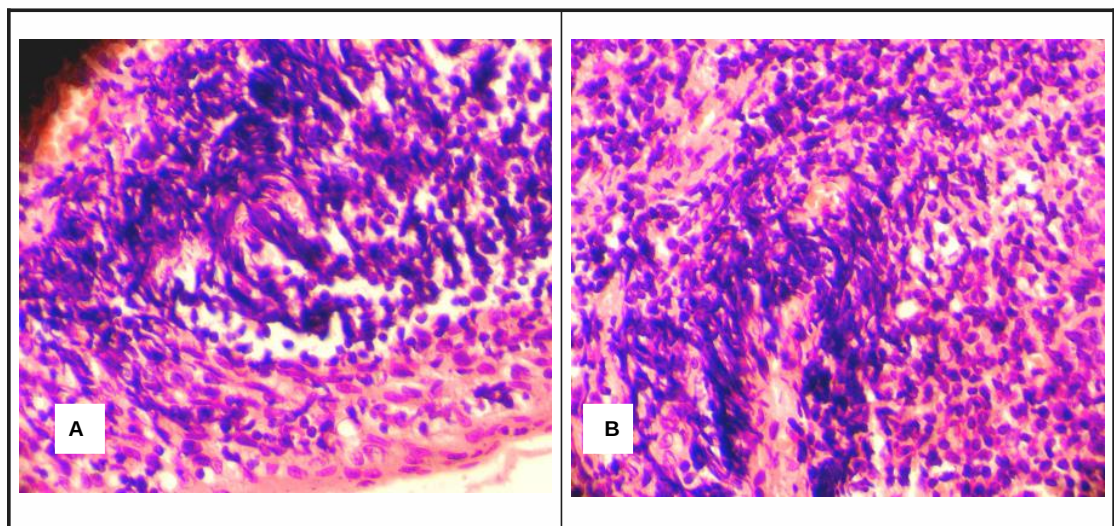


Fig.4.16: Damage of tissue in tonsils harbors *S.pyogenes*. (A) Epithelium layer. (B) Extra follicular area.

5. Discussion:

5.1. Bacterial profile:

The microbiological studies of the tonsil core removed from recurrent tonsillitis patients show that *Haemophilus influenzae* was the most common bacterium isolated (23.3%), followed by mixture of *S.aureus* and *S.pyogenes* (22.2%), then *S.aureus* in 17.7%, *S.pyogenes* in 17.2%, mixture of *S.aureus* and *H.influenzae* in 12.7%, and undefined gram positive and gram negative bacteria in 6.6%.

Our finding was comparable to finding of Brook *et al.* (1995) that identified mixed flora as a predominant in the core of the tonsils. While a study of core microflora in chronic tonsillitis patients by Abhay *et al.* (2005) show that *S.aureus* in 22% was most common isolate followed by beta haemolytic streptococci in 18%.

Timon *et al.* (1990) and Gul *et al.* (2007) confirm that swab cultures taken from the tonsillar surface may not always reveal the real pathogen of the tonsil core and may grow only commensal organisms confirming the inadequacy of superficial tonsillar swab as indicator of treatment.

5.2. Phagocytes activity:

Neutrophils are known to play an important role in inflammatory responses by virtue of their ability to perform a series of effectors function that collectively represent a major mechanism of innate immunity against injury or infection . Recently, it has become obvious that the contribution made by neutrophils to host defense and natural immunity extends well beyond their traditional role as professional phagocytes (Ryu *et al.*, 2004).

NBT test is a non-specific cytochemical test of neutrophil cytoplasmic membrane function, and membrane changes may be induced in vivo not only by endotoxin but also by tissue breakdown products and acute phase proteins (Gordon *et al.*, 1975).

As shown in table (4.2) NBT reduction give a normal level in RT-TE for the three study groups and not exceeding the normal rang 1-11% (Freeman and King, 1972b).

Several factors explain these result including bacterial toxin, chronic stat, and modulation of phagocyte antibacterial activity by antibiotics.

For the first factor it has been reported that pretreatment of PMN with high dose (>10 hemolytic units) of *S.aureus* alpha-toxin result in reduction of PMNs phagocytic function (Gemmell *et al.*, 1982). On the basis of this study, we hypothesize that with recurrent infection, *S.aureus* alpha-toxin enhanced in mucosal tissue (tonsils) and transfer through concentration gradient to systemic circulation leading to remain the phagocytic activity with normal level.

Further more, a direct suppression of neutrophil function by coagulase has been postulated (Aarestrup *et al.*, 1994).

Also normal NBT reduction value was observed in tonsils harbors *S.pyogenes* or mixed bacteria and this may related to toxin secreted by this bacteria which prevent PMNs stimulation.

Paton and Ferrante (1983) show that pneumolysin secreted by *S.pneumoniae* inhibit human PMN respiratory burst, bactericidal activity, and migration.

Several reports on chronic infection (The second factor) support our result. Walker and Urbaniak (1980) demonstrated that patient with chronic mucocutaneous candidiasis had a normal NBT reduction value.

Continuous using of antibiotic (Third factor) by recurrent tonsillitis patient directly alter the phagocytic function including interference with myelopoeisis leading to detrimental effect such as neutropenia, which usually take 1-2 weeks to be expressed and are not dose dependent. In susceptible patients, onset may occur within 24 to 48 hr. of starting the therapy penicillin G (the first choice antibiotic for RT patient), are the antibiotic most frequently involved in these deleterious events. This antibiotic is non-cell-penetrating drugs and enables to destroy intracellular pathogens. Erythromycins (the second choice antibiotic for RT

patient) have been proposed for binding to intracellular target and also have a depressed effecting on chemotaxis and oxidative burst (Therese-Labro, 2000).

Strong resistance of group A streptococcus to phagocytosis related to factor H and fibrinogen binding M protein and disarming complement component C5a by the C5a peptidase (Cunningham, 2000), and survival and persistent of *S.aureus* with PMNs (Therese-Labro, 2000) suggest the recurrent infection in tonsillitis patients.

Age had no significant effect on neutrophil function among different age groups, while the healthy controls show significant differences between first (5-9 yr.) And last age group (20-30 yr.) for neutrophil function and this may attribute to fact that immune system under normal condition became more effective against the foreign substance with the age of adults in compare to children.

5.3. Leukocyte migration factor:

Leukocyte migration factor was originally described as a T cell product and was later found to also be generated by pituitary cells, macrophages, eosinophils, tubular epithelial cell in kidney and epithelial cell in lung (Riedemann *et al.*, 2004).

It thought to be effectors molecules responsible for the expression of both humoral and cellular immune reaction. Some lymphokines may exert their positive or negative effects on their target cells. For example, the chemotactic factors and migration-stimulation factors increase random or cell-direct movement of cells, whereas the migration inhibitory factors retard random or cell-direct movement (Meshulam *et al.*, 1982).

The patient with an inhibition of cell migration considered more sensitized than patients with indices indicating stimulation (Soborg & Bertram, 1968).

Systemic LIF show an inhibition for all bacteria in first age group, puberty and adolescent show stimulation and inhibition while adult patient show stimulation for all bacteria (Table 4.4).

In children both cellular and humoral immunity induced directly and show a response and inhibition of migration depend upon the degree of cell sensitivity to pathogenic antigen. He *et al.* (2006) concluded that children aged 5-9 years show a significant increase in cellular immunity through increased IFN-gamma + CD4 and CD8 T cells after live attenuated influenza vaccine (LAIV).

Same result for direct stimulation of IgG antibody against Pneumococcal vaccine in infants was reported by Lottenbach *et al.* (1999).

Puberty and adolescent show a different immune response, evidence suggested that immunological responses and sex steroid hormones (Oestrogen and Androgens) are affected by each other (Dasilva, 1999), thus steroid hormone and sex (male or female) among the factor which may explain the varying of LIF between stimulation and inhibition among this group.

Globally, estrogens depress T cell dependent immune function but enhance antibody production. While androgen suppress both T cell and B cell immune responses (Silva, 1999).

Adult person show stimulation for all bacterial groups and this may refer to less sensitization to pathogenic antigen. This finding is consistent with He *et al.* (2006), showing that cellular immunity including IFN-gamma +CD4 and CD8 T cells was not increased after live attenuated influenza vaccine in adult person.

Furthermore, Lottenbach *et al.* (1999) reported that IgG antibody was not enhanced in responses to Pneumococcal vaccines in adult person.

Systemic LIF in control groups (table 4.5) show intermediary degree of sensitivity gave neither inhibition nor stimulation which explained through absent of specific sensitized lymphocytes in the blood of control groups to bacterial antigens used in vitro as sensitizer.

Comparison between systemic LIF in RT-patient and control groups (table 4.6) show that systemic LIF in RT-patient was inhibited nearly in all age group for all bacteria in compare to control groups. This explain through the presence of their specific sensitize antigen in vitro. Our explanation was come with the results obtained by Soborg and Bertram, 1968 ; Read *et al.*, 1974 which show that non-

sensitive controls were all grouped around an index of 1.00 with minor fluctuation while patient with acute rheumatic fever or uncomplicated streptococcal infections exhibit cellular reactivity to different streptococcal antigens result in an inhibition of migration.

Mucosal LIF show stimulation for all bacteria in all age group, during acute bacterial tonsillitis the bacterial antigen either *S.aureus* or *S.pyogenes* or mixed bacteria processed and presented to T_H cell leading to activation of T cell and secretion of LIF. With recurrent infection and enter of patient to chronic state antigen induce suppression and mucosal tolerance phenomenon developed leading to inhibition in T cell activity and reduce LIF as a result increase in migration index (table 4.7). Our explanation was come with the results obtained by Calandra *et al.* (1994) which show that LIF secretion was induced by as little as 10 pg/ml of lipopolysaccharide (LPS), peaked at 1 ng/ml, and was undetectable at LPS concentration > 1 µg/ml.

As shown in table 4.8, mucosal LIF show an increased stimulation in compare with systemic LIF. But because few point altered migration index, (MI >1 stimulation, and MI <1 inhibition), we can't considered *S.aureus* & *S.pyogenes* alteration not significant, in such case Ganong (2001) insisted on replacement of evaluation by subjective impression with analysis by statistical methods and considered it as important goal in medical sciences.

Systemic LIF with a wide area indicating a stimulation as well as an inhibition, and mucosal LIF with stimulation for all bacteria in all age group consistent with the fact that MI depend upon contact with Ag, concentration of Ag, and degree of sensitivity of cell donor.

5.4. E-rosette formation:

CD2 is a lymphocyte surface glycoprotein of 50-58 KD that appears early in thymocyte ontogeny and is present on all mature T cells. Rosetting of human T cells with erythrocyte is due to binding of CD2 to lymphocyte function-associated antigen-3 (LFA-3) on the erythrocyte (Plunkett *et al.*, 1987).

As shown in table (4.9) no significant differences in rosette-formation among different bacteria in RT-patient was observed. However significant increase in systemic E-rosette was observed in compare to age mach control group. Stimulation of human lymphocytes by bacteria is supported by the observation that patients with bacterial infection have higher than normal spontaneous blast transformation in peripheral blood lymphocytes (Wagnerova *et al.*, 1975).

This significant increased in systemic E-RFC is slight in compare to acute infection, which may reach 62% in brucellosis, 68% in typhoid fever, and 86% in infectious mononucleosis (Aiuti *et al.*, 1973). Thus we attribute this result to Chase-Sulzberger phenomenon of tolerance which suggested that development of mucosal tolerance consist with systemic immunologic hyporesponsivness (Ogra *et al.*, 2001).

Even though, Drucker and colleagues (1979) showed that proliferative responsiveness of blood lymphocyte to mitogen or bacterial antigens was markedly increased after tonsillectomy suggested existence of correlates of cellular immunity to bacterial products in the mucosal surface. In additions, it's proposed that tonsils may possess immunosuppressive activity for peripheral blood lymphocyte which may be related to local tonsillar infection.

On the other hand, tonsillar tissue lymphocytes show an inhibition in rosette formation especially in those harbors *S.aureus* and mixed bacteria in their tonsils as shown in table (4.10). These observations suggest that continuous background stimulation of tonsillar tissue lymphocytes may increase their unstimulation proliferative activity while decreasing their sensitivity to other additional antigenic challenge. This phenomenon (antigen-induced suppression)

may be due to stimulation of suppressor T-cell or competition in the processing of antigens by macrophages. It is well documented that bacterial stimulants of immune system may also act under different conditions, as immunodepressants or the lymphocytes from diseased tonsils could be refractory or tolerant to bacterial activator (Jesic *et al.*, 2005).

Further more, Chevrier and colleagues (1982) concluded that hyaluronic acid (group A streptococcal capsule) have an immunosuppressive activity for rosette formation.

Nearly, a significant decrease was detected in mucosal E-rosette in compare to systemic especially in tonsils harbors mixed bacteria (table 4.11) several possible pointes might be involved: first, tonsillar immunoglobulin secretion differs from the usual mucosal associated lymphoid tissue pattern in which IgG-producing cells predominate in spite of IgA. Decock *et al.*, (1978) concluded that aggregated IgG significantly inhibited E-rosette formation of T lymphocytes. The second possibility is that membrane markers maybe blocked by antigen-antibody-complement complex (Daniele *et al.*, 1975) and the third possibility is that lymphocyte population at an early developing stage bears insufficient density membrane markers to be detected by the techniques described (Gelfand *et al.*, 1974).

Gyenyey *et al.*, (1978) concluded that frequency of acute tonsillitis with clinical symptoms of chronic local inflammation decrease percentage of T cells.

Interestingly, age had no significant effect on activation of lymphocyte in chronic state for all bacteria in systemic immunity (Table 4.12). While mucosal immunity show a different effect of age in rosette formation (Table 4.13).

Reports related to effect of age on activation of lymphocyte are very limited. However, there is evidence that both systemic & mucosal E-rosette in recurrent state affected by the nature of antigen and concentration of antigen (Calandra *et al.*, 1994), antibiotic therapy (Therese-Labro, 2000), number of lymphocyte and body temperature (Hill *et al.*, 1974).

5.5. Specific humoral immune response in both systemic and mucosal:

Palatine tonsil is a B-cell dominant lymphoid organ with important role in specific immunity. Immunoglobulins appear to be the result of B-cell activation and differentiation, and they are also indicator of tonsillar tissue immunoreactivity (Avramovic *et al.*, 1998).

Specific Ig titer in both systemic and local show a different titer among the patient. This discrepancy for the titer could be explained by the variable systemic immune response and variable mucosal immune response in each analyzed tonsils, especially with regard to degree of fibrosis as a consequence of chronic inflammation.

Also the data indicated a low increase to normal titer for specific antibody in the serum of RT-patient in compare to control group, while the tonsils show a higher titer for specific antibody. This conclusion is in keeping with earlier reports that show that intranasal immunization and local tonsillar infection prime tonsillar lymphocytes more efficiently than dose parenteral vaccination (Morag *et al.*, 1975).

Similarly oral immunization was poorly effective at inducing an antibody response in tonsils in contrast to local (intra tonsillar) immunization. The fact that tonsillar immunoglobulin-secreting cell (ISC) are induced to produced mainly IgG (Brandtzaeg *et al.*, 1978), whereas intestinally activated antibody secretion cells preferentially secrete IgA (Quiding-Jarbrink *et al.*, 1995) suggesting that tonsillar immune system functions independently not only from systemic immune apparatus, but also from lower interomucosal immune system.

Specific antibody titer, although show slight increase related to chronic state, was higher in RT-patient harbors *S.aureus* in compare to those harbors *S.pyogenes* or mixed bacteria. This result was consistent with our histological finding.

S.pyogenes depends on its ability to avoid IgG and interfere with IgFc-mediated phagocytosis through streptococcal cysteine proteinase (IdeS) which specifically cleaves the heavy chain of IgG as a specific strategy for survival.

Akesson *et al.* (2006) confirmed that antibodies against IdeS appear not to be directly affecting the outcome of invasive disease and not protect the patient from recurrent disease. In contrast, Aoki *et al.* (1987) confirmed that IFN- γ might specifically exert proliferative effect on B-cell lineage obtained from human tonsils specifically in *S.aureus* infection.

Interestingly, the major effector cell in the mucosal surface are not B-cells but T-lymphocyte of CD4⁺ as well as CD8⁺ phenotypes (Ogra *et al.*, 2001).

When B lymphocyte or Th2 lymphocyte came across the *S.aureus* epitopes and *S.pyogenes* epitopes, the S.A.E is either damp the reactivity of lymphocytes to S.P.E or it heightens it or doesn't affect it. Same thing for S.P.E thus the outcome of specific antibody for mixed infection can be high for S.A.E and low for S.P.E, high for both or low for both (Zubler, 1998). Thus the observed serum and mucosal antibodies titer can be a net result of the antigen competition process in vivo or the antigenic competition between *S.aureus* and *S.pyogenes* patient sera as well as mucosal in vitro (Shnawa & Mehdi, 2004 ; Taussig, 1975).

5.6. Cytokines (IL-6, IL-10, and IFN-gamma):

Palatine tonsils, a major component of lymphoid tissue in Waldeyer's ring, were chosen for characterization of cytokine production. Histological and lymphocyte studies show that this tissue belongs to so-called mucosa-associated lymphoid tissue (MALT) and appears to function as a first line of defense against microorganisms.

Cytokines including interleukin-2 (IL-2), IFN- γ , tumor necrosis factor- β (TNF- β) and IL-6 has been previously reported in tonsils obtained from children with infectious mononucleosis (IM) (Andersson and Andersson, 1993). However, this study was performed to analyze cytokine production within the tonsils among RT-patients.

Interleukin-6 is a cytokine derived from activated T lymphocytes that has many functions, including induction of B-cell growth; induction of B-cell differentiation and antibody production; induction of differentiation and

proliferation of T cells; synergistic induction with IL-3 of hematopoietic cell growth; and induction of hepatocyte secretion of acute-phase inflammatory proteins (Parslow *et al.*, 2001).

Our results indicated a low concentration of IL-6 in the serum of RT-patient. This finding is consistent with the recent finding of Passali group (2004) which indicated a low concentration of IL-6 in tonsillitis patient to reach $(2.1 \pm 1.2 \text{ pg/ml})$.

No differences in IL-6 concentration was observed in the mucosal of RT-patients among those harbors *S.aureus* or *S.pyogenes* or mixed bacteria in their tonsils (table 4.19). This finding is consistent with Murakata *et al.* (1999) concluding that there is no significant differences in cytokine production by tonsillar mononuclear cells among pustulosis palmaris et plantaris (PPP) and non (PPP) subjects, when stimulated with *S.pyogenes* and *S.aureus* bacteria.

Further more, IL-10 has been reported to inhibit the release of proinflammatory cytokines such as IL-6. Waal-Malefyt and colleagues (1991) show that administration of $1 \text{ } \mu\text{g/ml}$ of LPS to human monocyte culture induce production of IL-6 to reach 261.7 ng/ml while combination of LPS with $100 \text{ } \mu\text{g/ml}$ IL-10 reduce IL-6 concentration to reach 78 ng/ml .

IL-10, which recognized for its ability to inhibit activation and effectors function of T-cells, monocytes and macrophages, show a significant increase in the serum of RT-patient in compare to control group ($59.391 \pm 8.044 \text{ ng/ml}$) especially those harbors *S.pyogenes* in their tonsils to reach $99.780 \pm 5.640 \text{ ng/ml}$ (Table 4.19).

Tonsils which is a highly active immunological organs containing large number of T-cells spontaneously producing IFN- γ .

Our data show a normal level for IFN- γ , this may be explained through the fact that RT-patient were tonsillectomized when healthy, and had not received antibiotics. Thus the cytokine patterns found most likely reflects a homeostatic Balance. One may speculate that a more polarized cytokine production profile would have been detected if the tonsils tissue was obtained during exacerbation.

Table (4.20) indicated that human tonsils are sites of continuous lymphoid activation, as demonstrated by high cytokine concentration. This reflects the constant stimulation by environmental antigens encountered in the oropharyngeal cavity. Both Th1 and Th2 types of cytokine responses were expressed in tonsils tissue (Andersson *et al.*, 1994). However, for most RT-patient IL-10 concentrations exceeded that of IFN- γ and IL-6 concentration.

In general, our data show that systemic and mucosal IL-6, IL-10, and IFN- γ were found in higher concentration in RT-patient harbors *S.pyogenes* in their tonsils (Histologically associated with atrophy or mild hypertrophied tonsils) in compare to those harbors *S.aureus* in their tonsils (Histologically associated with hypertrophied tonsils) this data are consistent with recent immunohistochemical findings from Agren's group(1996), which suggested that IL-2, IL-6, IL-10, and IFN- γ were found much more often in section obtained from RT tissue compared with those from hypertrophied tonsils.

On the other hand, surface M proteins which act as a super antigen (Tomai *et al.*, 1990) was specifically stimulates T cells expressing V β 2, V β 4, and V β 8 elements, thus induced IFN- γ production (Kotb *et al.*, 1993).

Table (4.21) show that RT-TE harbors mixed bacteria in their tonsils show a significant decrease in mucosal IFN- γ to reach 1.198 ± 0.100 IU/ml in compare to those harbors *S.pyogenes* or *S.aureus* in their tonsils (2.286 ± 0.134 IU/ml, 2.071 ± 0.183 IU/ml). This may explain through the decrease in Th1 cells stability for cytokine expression under the presence of different antigen (mixed bacteria). This converting condition was also described by Assenmacher and colleagues (1998).

The high tissue concentration of IL-6, IL-10, and IFN- γ is an expression of local hyper production or overproduction, due to monocyte-macrophage activation, T cell activation, and B cell activation caused by repeated stimulation by the pathogenic agents. The presence of high levels of these cytokines in mucosal. Nevertheless, instead of inducing an increase in immunological efficiency may have a damaging effect on these mechanisms in the long term.

These mediators, in fact, induce the activation and proliferation of endothelial cells and fibroblasts, which may result, with time, in the progressive replacement of immunologically active tissue with fibrotic tissue.

5.7. Histological changes:

A palatine tonsil is a part of mucosal lymphoid system play role in the first-line defense system of the body. They are composed of B-lymphocytes arranged in follicles, T-lymphocytes and macrophages located in the surrounding tissue. B-lymphocytes responsible for synthesis of different immunoglobulin mostly IgG, especially with polyclonal activators such as different bacteria (Jesic *et al.*, 2005).

5.7.1. Tonsillar crypts and epithelium:

Lymphocyte traffic is essential because only few immunocompetent cells specific for an individual antigen are available in a non-affected tonsil (Nave *et al.*, 2001).

Migration of lymphoid cells is strictly regulated by the expression of multiple adhesion molecules and receptors for chemo attractants (Chemokine receptors) that interact in a tissue-specific manner with corresponding ligands on endothelial stromal cells. The extravasations of mainly nave T and B cells in to inductive lymphoid compartments takes place through specialized post capillary so-called high endothelial venules (HEV), and is largely regulated in a similar manner in the systemic and mucosal immune system (Brandtzaeg, 2003).

The crypt epithelium of the palatine was reticulated in all the subjects examined. It was a modified form of stratified squamous epithelium, which contained lymphocytes, plasma cells, macrophages and interdigitating cells. These cell types are known to be present in the crypt epithelium as early as the 15th week of gestation, which suggests that reticulation is a normal developmental event, and occurs concurrently with the formation of primary follicles, interfollicular area and HEVs (Perry, 1994).

The main structural features of the crypt epithelium were, first, the epithelial cells, altered in shape but still representing its building blocks ; second, the presence, and in some areas the predominance, of non epithelial cells ; and third, the intraepithelial location of numerous vessels, often extending throughout the epithelial thickness.

The infiltration of the tonsillar crypt epithelium with non epithelial cells was not uniform, resulting in a patchy reticulated appearance. When it was modified to such a degree that the characteristics ascribed to an epithelium was lost, it presented the appearance of a functional lymphoid compartment (Brandtzaeg, 1988).

These reticulated patches were interspersed with patches of stratified squamous epithelium, which contained only a few infiltrating cells. Presumably the sites of the reticulated patches in any individual tonsil were not predetermined, but varied with time and depended on the degree of the current antigenic stimulation.

The direct transepithelial access of antigens result in greater influx and recruitment of non epithelial cells in a particular patch, in contrast to a less strongly stimulated area which may have retained stratified squamous non keratinized cover.

Epithelial barriers on mucosal surfaces at different sites in the body differ dramatically in their cellular organization and thickness (Neutra *et al.*, 1996).

Tonsils harbors *S.aureus*, *S.pyogenes* or mixed bacteria show thickened and inflamed epithelial surface in compared to normal tonsils. However *S.pyogenes* or mixed bacteria show more thickened and inflamed epithelial surface. This refers to the hallmark of extra cellular product (invasins) which have irritative effect on epithelial cells.

The right balance of the number of epithelial and non epithelial cells in the reticulated crypt epithelium might be vital for the preservation of efficient mucosal protection. The locally produced secretory IgA (sIgA) molecule is stabilized and transported by the secretory component in the cytoplasmic

membrane of epithelial cells, which synthesis this polypeptide (Brandtzaeg, 1984; Konig *et al.*, 1987).

This transport is unique in that the secretory component is not recycled, but must be continually re-synthesized by the epithelial cells. Thus a sufficient number of competent epithelial cells must be present in a reticulated patch in order to retain this function.

In some patches the infiltration almost reached the upper most stratum and the epithelial surface, provided by only 1 or 2 cell layers, remained intact. This type of epithelium described as the 'lympho-epithelial symbiosis' acquired the appearance and presumably also the role of a lymphoid characteristics. However, it still maintained a cover and thus protected the internal environment of the tonsil from the external environment of the crypt lumen.

Many IgA plasma cells must develop daily from B cells to guarantee a continuous supply of IgA antibodies in the air way mucosa. For this, the surrounding cell must provide a constant supply of cytokines necessary for B-cell isotype switch, growth and differentiation into IgA-secreting plasma cells (Salvi & Holgate, 1999).

The distribution of plasma cells in the surface epithelium shows a reduced number in all studied groups. Based on the quantification of Ig-producing cell, Avramovic *et al.* (1998) reported that Ig-producing cells were predominantly associated with the crypt epithelium and subepithelial region and that variable number was found in extrafollicular areas and in germinal centers of the lymphoid follicles. Olofsson *et al.* (1998) also reported the absence of B cell in the surface epithelium. While, Nave *et al.* (2001) confirmed that plasma cells are predominantly located around intraepithelial capillaries.

Our observation concerning neutrophils include the migration of neutrophil to tonsillar surface secretion, absence of neutrophils in tonsillar parenchyma and a significant decrease in neutrophils in tissue harbors *S.pyogenes*.

As no bacteria were observed in the tonsillar epithelium, the migration of neutrophils to the epithelium could not be explained by neutrophil migration to invading bacteria. Instead, presence of functionally active neutrophils in the secretion on the tonsillar surface suggest a steady active migration of neutrophils to the secretion on the tonsillar surface as a part of an active cellular defense and this obtains support by Ebenfelt and Ivarsson(2001) finding which indicated an active physiological migration of neutrophils to the tonsillar surface.

Infection is usually defined as a bacterial invasion and the host's response to that invasion, the location of an infection should be the place where the neutrophils attack and phagocytes the bacteria. Our histological results shown neither bacterial invasion nor phagocytes of bacteria occur in the tonsillar tissue of RT patients. It is the tonsillar surface secretion that bacteria occur, and are attacked and phagocytes by the neutrophil. This mean that infections process occur in the secretion on the tonsillar surface and not in the tonsillar parenchyma. This observation has been supported by Ebenfelt (2002) finding. If there had been an invasion of bacteria in the tonsillar parenchyma and an inflammatory response to that invasion, one would have expected not only to observe bacteria but also-if bacteria were over looked-a distribution of neutrophils to all tonsillar area, even the germinal centers.

No migration of PMN or PMN in tonsillar surface secretion was observed in RT patient harbours *S.pyogenes* in their tonsils. This may explain through streptococcal virulence factor C5a peptidase which destroyed the chemotactic signal generated by the alternative complement pathway, there by inhibiting recruitment of PMN to the site of infection (Therese-Labro, 2000).

5.7.2. Extra follicular area:

Our microscopic studies clearly showed that many macrophage and dendritic cells are in close contact with lymphocytes and plasma cells. The interaction between APC and plasma cells suggested that such interaction may not responsible for antigen presentation but may be coordinating sign of regulation between humoral and cellular immunity.

Extra follicular region in RT patient harbors *S.aureus* show a density of plasma cell in compare with those harbor *S.pyogenes* and mixed bacteria, suggesting that *S.aureus* can stimulate IFN-gamma to enhance or induce proliferation of B-cell, this information can be interpreted in several ways. Firstly, IFN-gamma might have B-cell growth factors activity leading to enhance proliferation of activated B cells through the IFN-gamma receptor expressed on the cells. Secondly, IL-1 might be involved in the observed enhancement of the B-cell response, considering that IL-1 can be produced by B-cells as reported (Matsushima *et al.*, 1985) and IFN-gamma could augment IL-1 effects by enhancing IL-1 release (Newton, 1985). Thirdly, other as yet unidentified molecules distinct from IL-1 might be induced by IFN-gamma and thereby contributes to the enhancement of the B-cell response by functioning as B-cell stimulating factors. Finally, it cannot be entirely excluded that there was an enhancement of IL-2 effects through IL-2 receptors on B cells (Tsudo *et al.*, 1984).

5.7.3. Follicular area:

In this study two types of histological change were noticed. The first type represents the hypertrophied tonsils with large number of follicles with bright and active germinative centers. On the other side there are atrophic tonsils with only one or two dark follicles. This alternation features may attribute to recurrent infection in which recurrent infection produce a possible immunological tolerance to antigen stimulation and could lead to the tonsillar hypertrophy. Thus

hypertrophy could be adaptive mechanisms in term of improvement of immunological function destroyed by recurrent infection (Jesic *et al.*, 2005).

Follicular dendritic cells within the germinal centre retain immune complexes that select the developing B cells for which they have a higher affinity. Subset of dendritic cells germinal centers are strong antigen presenting cells for T cells, but not co-stimulate CD40 activated B cells. These dendritic cells probably stimulate germinal centre T cells and aid the processes that are required for the generation of memory B-cells.

Cellular necrosis and tissue damage was observed in RT harbors *S.pyogenes* in their tonsils. This damage can be attributing to sever invasive activity of *S.pyogenes* through secretion of a large extra cellular product, invasins, which include: Streptokinase (fibrin lysis), Streptodornase A-D (possesses deoxyribonuclease activity, B and D ribonuclease activity), Hyaluronidase (digest host connective tissue hyaluronic acid), Protease (cause soft tissue necrosis), Streptolysin (S and O is leukocidin). Even so, antibodies to these products are relatively insignificant in protection of the host.

Further more, The capsule, M-protein, peptidoglycan, N-acetylglucosamine, group specific carbohydrate, portions of the cell surface and cytoplasmic membrane all have antigenic epitopes that mimic those of mammalian muscle and connective tissue thus resulting in tolerance or suppressed immune response (Cunningham, 2000 ; Cywes *et al.*, 2000 ; and Heath *et al.*, 1999).

On the other hand, *S.aureus* invade the tonsils with less number of invasins (in compare to *S.pyogenes*) giving opportunity for immune response to activated. With recurrent infection endothelial cells and fibroblasts activated and proliferated and in most tonsils harbors *S.aureus* result in fibrotic tissue and hypertrophied tonsils. Same result for prevalence of *S.aureus* in hypertrophic tonsils was reported by Kuhn *et al.* (1995) and Ramirez *et al.* (1997).

Conclusions

1- Systemic immune response shows leukocyte migration inhibition, while tonsillar immune response shows stimulation related to tolerance phenomenon and antigen induced suppression. However, systemic E-rosette forming T lymphocytes were elevated in comparison to tonsillar E-rosette forming cell.

2- Systemic Ig titer shows a normal level, while mucosal Ig titers show a higher level than that of systemic.

3- Normal IL-6 and IFN- γ was observed in systemic immune response with significant increase ($P < 0.001$) in IL-10 concentration, while mucosal IL-6, IL-10, and IFN- γ was highly increased.

4-Age between 5-30 yr. had no significant effect on systemic NBT and E-rosette, while LIF and mucosal E-rosette harbors *S.aureus* in their tonsils indicated that children (5-9 yr.) are more sensitive to pathogenic antigen than adults (20-30 yr.).

5- Tonsillar *S.pyogenes* effect on cellular organization, thickness of epithelium surface, connective tissue digestion, fibrin lysis, and necrosis. Meanwhile *S.aureus* induce an enhancement of the numerical density of plasma cell in extrafollicular area.

6- Migration of PMN cell and PMN in tonsillar surface secretion was observed in RT-patient harbors *S.aureus* and mixed bacteria in their tonsils such pattern was not observed in tonsils harbors *S.pyogenes*.

7- Recurrent tonsillitis patient harbors *S.aureus* in their tonsils show increased number of follicle, defined germinal center borders, distinct morphological

zones and frequently hypertrophied, while patients harbors *S.pyogenes* in their tonsils show more tissue damage, necrotized, atrophied to mild hypertrophied, with less number of follicle which had germinal center with ill-defined borders.

Recommendations

- 1- Study the immune response among RT-patients harbors gram negative bacteria in their tonsils specially *Hemophilus influenzae*.
- 2- Study the immunohistochemical aspects using appropriate method for quantification of antibody and cytokines which give information on the dynamic aspects of immune responses and precise anatomical locations of antibody or cytokine formation with an emphasis on IL-4 determination.
- 3- Study the immune parameter for chronic recurrent tonsillitis patients before and after tonsillectomy.

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Appendix

Measurement the diameter of the follicle and numerate the number of cell in mm^2 .

A/ The way for measurement follicular diameter:

- 1- Calibrate ocular micrometer with stage micrometer, rotate the eye piece until the tow scales was parallel and ocular coincide precisely with a line of the stage scale.

Suppose that 38 units on the ocular scale equal 10 lines on the stage micrometer at 40x. (The distance between divisions of stage micrometer was 0.01).

$$38 \text{ ocular units} = 10 * 0.01$$

$$1 \text{ ocular units} = 0.1/38 = 0.0026315 \text{ mm}$$

$$1 \text{ mm} = 1000 \mu\text{m}$$

$$\text{Therefore } 1 \text{ ocular unit} = 0.0026315 \text{ mm} = 2.6315 \mu\text{m}$$

B/ The way for numerate lymphocytic cell, antigen presenting cell (APC), and plasma cell / mm^2 :

- 1- Using stage micrometer calibrates the diameter of the field (The distance between divisions of stage micrometer was 0.01).suppose that diameter 20 line on stage micrometer at 100 x.

$$\text{Diameter of the field} = 20 * 0.01 = 0.2 \text{ mm}$$

$$\text{Area of the field} = 0.1 * 0.1 * 3.142 = 0.0314 \text{ mm}^2$$

$$\text{Suppose that number of cell was 5 in } 0.0314 \text{ mm}^2$$

$$\text{Thus number of cell/1 mm}^2 = (5 * 1) / 0.0314 = 159.13 \text{ cell/mm}^2$$