An Evaluation Study Of Cellular Immunological Functions In Anergic Tuberculous Patients

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Dedication

To the memory of my mother…With my endless sorrow

To my father… brothers and sisters with my endless love

Mohammed

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 requirements for the degree of Doctor Philosophy in Biology (Microbiology) and this work has never been published anywhere.

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ABSTRACT

Tuberculosis remains the most common infectious disease in the world and is one of the leading cause of death worldwide. TB-associated anergy makes several diagnostic and theuraptic problems. The present work aimed to evaluate the cellular immunological parameters in TB patients and try to make clear-cut differences among anergic TB patients (AN), allergic TB patients (AL), and control subjects (C). The work was carried out on ١٢٥ TB patients and ٢٣ apparently healthy controls. TB patients consisted of two groups, AN patients ٨٢ (٤٧ males and ٣٥ females) and AL patients ٤٣ (٣٢ males and ١١ females). *In vivo* specific and non-specific, as well as *in vitro* specific and non-specific parameters were attempted to evaluate the cellular immune state of TB patients. *In vivo* specific parameters were tuberculin skin test, detection of BCG scars, and AFB; while *in vivo* non-specific parameters were demonstration of the pattern of the local and systemic leukocyte response. E-rosette and LIF were used as *in vitro* specific parameters, while the *in vitro* non-specific parameter was represented by NBT test.

The results of systemic leukocyte response revealed that neutrophil was the predominant leukocyte type in both patients groups followed by lymphocyte, monocyte, eosinophil, and basophil. Eosinophil and basophil showed an increasing degree in AL when compared to AN and C, whereas local leukocyte response was characterized by the wide median value of neutrophils and lymphocytes in AN in comparison to that of AL. NBT test showed gradual differences among the three studying groups, the NBT median values were ٤.٠٪, ٧.٠٪, and ١١.٠٪ in AN, AL, and C respectively. E- rosette test showed clear differences among the three groups, the median values were ١٤.٠٪, ٢١.٠٪, and ٢٥.٠٪ respectively, such gradual variations were noted in the LIF median values, which were ٥٧.١٤٪, ٣٧.٥٪, and ٨٨.٢٪. BCG scars were noted at high rate in AL and C when compared to AN.

AFB were microscopically present at low rate in both patients groups. The two tailed t-statistics for the inter-group differences in the NBT, E-rosette, and LIF tests were significant at P=٠.٠٠١ level. Thus indicating the validity of putting such tests in evaluation of anergic tuberculous patients.

From the results of this work, one could conclude that E- rosette, LIF and to a lesser extent NBT can be used as cellular parameters to judge the immune response of TB patients whether of anergic or allergic form.

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Abbreviation	Meaning
ADCC	Antibody dependent cell mediated cytotoxicity
AFB	Acid fast bacilli
AG	Arabinogalactan
AIDS	Acquired immunodeficiency syndrom
AL	Allergic tuberculous patients
AN	Anergic tuberculous patients
APCs	Antigen presenting cells
AP-١	Transcription factor activating protein-١
BCG	Bacillus-Calmette-Guérin
bcl-٢	An inhibitory gene of apoptosis
B٧	Co-Stimulatory protein expressed on APCs
C	Control
CBMCs	Cord blood mononuclear cells
CD	Cluster of differentiation
CMI	Cell-mediated immunity
CR	Complement receptor
CSF	Colony stimulating factor
CTLA-٤	T-cell surface molecule induced on activation
DLC	Differential leukocyte count

DNA	Deoxyribonucleic acid
DTH	Delayed-type hypersensitivity
dTTP	Deoxy thiamine triphosphate
EAC	Erythrocyte-antibody-complement-rosette
ELISA	Enzyme linked immunosorbent assay
E-rosette	Erythrocyte- rosette
E-Selectin	Adhesion protein present on leukocytes
Fc	Crystalline fragment of immunoglobulin molecule
G+C	Guanine and Cytosine percent
Go	Interphase of cell cycle
GRAIL	A gene related to anergy in lymphocytes
□δ T cells	Gamma-delta T cells
HIV	Human immunodeficiency virus
HLA-DQ	Human leukocyte antigen-DQ allele
H_2O_2	Hydrogen peroxide
ICAM-۱	Intercellular cell adhesion molecule-۱
IFN□	Interferon-gamma
IFN□-R	Interferon-gamma receptor
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL-۱	Interleukin-۱
IL-۲	Interleukin-۲

IL-٤	Interleukin-٤
IL-٨	Interleukin-٨
IL-١٠	Interleukin-١٠
kDa	Kilo-Dalton
LIF	Leukocyte migration-inhibitory factor
MHC	Major histocompatability complex
MIF	Macrophage migration inhibitory factor
MMRc	Macrophage mannose receptor
M. tuberculosis	*Mycobacterium tuberculosis*
M.W.	Molecular weight
$MyD_{٨٨}$	Myeloid differentiation protein ٨٨
NADP	Nicotinamide adenine dinucleotide phosphate-oxidase
NADPH	Reduced adenine dinucleotide phosphate-oxidase
NBT	Nitrobluetetrazolium
NF-KB	One type of transcription factor of interleukin in lymphocytes
NK cells	Natural killer cells
No	Nitric oxide
Nramp ١	Natural-resistance-associated-macrophage protein ١
PCR	Polymerase chain reaction
PG	Peptidoglycan

PMNs	Polymorphnuclear leukocytes
PPD	Purified protein derivative
RBCs	Red blood corpuscles
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
S-IgA	Secretory-immunoglobulin-A
SRBCs	Sheep red blood corpuscles
TB	Tuberculosis
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
T_H cells	Helper T cells
TLRs	Toll-like receptors
TNF-α	Tumour necrosis factor-alpha
TNF- β	Tumour necrosis factor-beta
UTP	Uracil triphosphate
VCAM-١	Vascular cell adhesion molecule-١
VDR	Vitamine D receptor

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١-١ Overview:

To understand the immune state in vertebrates including humans during health and disease, one should first understand host biology, parasite biology, host-parasite balance versus imbalance and their outcomes. Thus, in this brief introduction. Mycobacteria, host, Mycobactria-host interactions, and their outcomes were mentioned and such a mentioning was mainly on human infected with *M. tuberculosis*, with an emphasis on an anergic tuberculosis.

١-٢ View to Mycobacteria:

The genus *Mycobacterium* is the only genus in the family Mycobacteriaceae and is related to other mycolic acid-containing genera. The high (G + C)content of the DNA of *Mycobacterium* species (٦١ to ٧١ mol %) except for *M. leprae* (٥٥ mol %) is within the range of those of the other mycolic acid-containing genera, *Gordonia* (٦٣ to ٦٩ mol %), *Tsukamurella* (٦٨ to ٧٤ mol %), *Nocardia* (٦٤ to ٧٢ mol %) and *Rhodococcus* (٦٣ to ٧٣ mol %) (Pfyffer *et. al.*, ٢٠٠٣). Lehmann and Neumann introduced the generic name “Mycobacterium” in ١٨٩٦. This name means fungus-bacteria was derived from the mold-like pellicular growth of Mycobacteria on liquid media (Grange, ١٩٨٨). Mycobacteria are Gram-positive, although they are not easily stainable by this method; aerobic, non-sporing, do not form capsules, and can be stained easily by acid fast stain, which represents the most characteristic properties of Mycobacteria (Brooks *et. al.*, ٢٠٠٤).

١-٣ Pathogenic Mycobacteria :

M. tuberculosis complex includes *M. Tuberculosis, M. bovis, M. africanum, M. microti* and *M. canetti*, which form a tight, discrete group of organisms that display >٩٥٪ DNA-DNA homology (Pfyffer *et. al.*, ٢٠٠٣).

***M. tuberculosis*:** *M. tuberculosis* had been identified as the causative agent of tuberculosis (TB) in ١٨٨٢ by Robert Koch (Fenton and Vermeulen, ١٩٩٦). It is one of the most ubiquitous pathogens in the world, and about one-third of the world's population is infected with *M. tuberculosis* and it is responsible for ٨-١٢ million cases of active TB each year and ٣ million deaths (Phalen and McMurray, ١٩٩٣; Raviglione *et. al.*, ١٩٩٥; Rojas *et. al.*, ١٩٩٩; Flynn and Chan, ٢٠٠١; Chackerian *et. al.*, ٢٠٠١; DesJardin *et, al.*, ٢٠٠٢; Smith, ٢٠٠٣; Bloomberg, ٢٠٠٤,a). Although several, Mycobacterial spp. can cause TB, *M. tuberculosis* is the principle causative agent (Goldsby *et. al.*, ٢٠٠٠). *M. tuberculosis* is regarded as the leading cause of death in USA and the number of TB cases has increased after ١٩٨٥ (National Academy of Sciences, ٢٠٠١).

M. bovis: It causes TB in warm-blooded animals such as cattles, dogs, cats, birds and humans. It is different from *M. tuberculosis* in that, *M. bovis* is resistant to pyrazinamide, which is regarded as the best effective drug against *M. tuberculosis* (Pfyffer *et. al.*, ٢٠٠٣).

M. africanum is a cause of human TB in tropical Africa.

M. microti is the causative agent of TB in Guinea pigs, rabbits, cats and it has been identified as a causative agent of TB in both immunocompetent and immunosuppressed humans. ***M. canettii*** is a causative agent of lymphoadenitis in children and of

generalized TB in human immunodefeciency virus (HIV)-positive patients (Pfyffer *et. al.*, ٢٠٠٣).

Leprosy is the second main disease caused by Mycobacteria, the causative agent is *M. Leprae*, which differs from all other mycobacteria in that it can not be cultured *in vitro* (Pfyffer *et. al*, ٢٠٠٣).

١-٤ Diagnosis of pumonary *tuberculosis:*

The diagnosis of TB is based on the following criteria:

* Clinical symptoms,
* Chest X-ray,
* Sputum smear,
* Cultivation of clinical specimes (mainly sputum), and
* Immunological tests (humoral and cellular).

١- Clinical symptoms:

Cough is the most common symptom of pulmonary TB, hemoptysis may rarely be a presenting symptom and does not necessarily indicate active TB. Genaralized symptoms include, weakness, fatigue, weight loss, and fever (Haselett *et. al.*, ٢٠٠٢).

٢- Chest-X-ray:

Pulmonary TB nearly always causes abnormalities on the chest film. In primary TB, occuring as a result of recent infection, the process is seen as a middle or lower lung zone infiltrate, often associated with psilateral hilar adenopathy. Cavitation may occur when cell mediated immunity develops (Dunlap *et. al.*, ٢٠٠٠).

٣- Sputum smear:

Sputum smears are examined for acid fast bacilli (AFB) by Ziehl-Neelsen staining. If acid fast organisms are found, this is presumptive (but not confirmed) evidence of TB infection, and confirmatory testing is therefore unavoidable (Laszalo, ١٩٩٩).

٤- Cultivation of *M. Tuberculosis:*

A definitive diagnosis of TB can be obtained only by culturing clinical specimens. Cultures of clinical specimens are associated with higher case detection rates, because the sensitivity of culture is much higher than that of smear microscopy. Sputum, and bronchoalveolar lavage fluid can be cultured on specific culture media (Löwnstein-Jenssen medium or Middlebrook ٧H١٠ with supplemented antibiotics). These solid media require three weeks or longer to show visible colonies. Liquid media (BACTC- ٤٦٠/١٤C- labeled palmitic acid) require ٢ weeks (Laszalo, ١٩٩٩). Biochemical methods can distinguish the cultured mycobacterial species depending on: (١) Phenotypic tests (Pigmentation, Niacin production, Nitrate reduction, urease production… etc), and (٢) Distinctive molecular characteristics of *M. tuberculosis*, which include two main methods: (a) Polymerase chain reaction technique (PCR), and (b) High performance liquid chromatography (Dunlap *et. al.*, ٢٠٠٠; Vincent *et. al.*, ٢٠٠٣).

٥- Immunological tests for tuberculosis:

Historically, the first immunodiagnostic test used in the identification of TB was the tuberculin skin test, but the shortcomings of this test include the inability to distinguish active disease from past

sensitization (Vincent *et. al.*, ٢٠٠٣). The tuberculin skin test has several drawbacks that reduce their sensitivity, this is due to: (١) False-negative result in case of pre-hypersensitive stage of infection; this test requires at least ٤ weeks after infection to give positive result, loss of sensitivity (anergy), AIDs, using of immunosuppressive drugs, and viral infection (measles infection), and (٢) False-positive result in case of immune reaction to BCG vaccine, and to nontuberculous mycobacteria (Layne, ٢٠٠٣;Khalifa *et.al.*,١٩٩١).

The specificity of serological tests with crude antigen preparation is too low for clinical application, but using purified antigens can increase this specificity. The antigens tested include the ٣٨-kDa antigen, lipoarabinomannan, antigen ٦٠, and glycolipids including phenolic glycolipid $Tb_{١,٢,٣}$- diacyltrehalose and lipooligosaceharide. These mycobacterial antigens can be tested serologically by using ELISA technique (Vincent *et. al.*, ٢٠٠٣).

Detection of changes in the level of serum immunoglobulins isotypes by using ELISA, reveals that IgG level increases during TB infection (Kardjito and Handoyo, ١٩٨٢). Likewise, using Mancini Lural agar diffusion (partigens), reveals that IgA level was mainly raises during pulmonary TB (Sawa *et. al.*, ١٩٨٦).

١-٥ Host factors and susceptibility to *Mycobacterium tuberculosis:*

Susceptibility to disease after infection by *M. tuberculosis* is influenced by environmental and host genetic factors. The progression of TB can have several outcomes, determined largely by the response of the host immune system. The efficacy of this response is affected by intrinsic factors, such as the genetics of the immune system as well as extrinsic

factors, e.g.; insults to the immune system and the nutritional and physiological state of the host (Smith, ٢٠٠٣). Layne (٢٠٠٣) showed that the factors contributing to spread of TB infections include: Ignorance, poverty, overcrowding, poor hygiene, war and economic depressions. Predisposing factors that enhance the reactivation to active TB disease include: HIV infection, drug and alcohol abuse, aging, malnutrition and diabetes (MCBIO ٢٠٠, ٢٠٠٤). Latent TB, as a malady can be viewed as an equilibrium between host and bacilli. In response to infection with *M. tuberculosis*, most persons mount a robust infection with *M. tuberculosis*, immune response that can be completely effective and kill all invading tubercle bacilli (Schluger and Rom, ١٩٨٨; Flynn and Chan, ٢٠٠١).

There is substantial evidence that host genetic factors are important in determining susceptibility to mycobacteria. Wilkinson *et. al.* (٢٠٠٠) showed that polymorphisms in the vitamin D receptor (VDR) gene contribute to increase the susceptibility to infection with *M. tuberculosis*.

Abnormalities in the gene for interferon gamma receptor (IFN ɣ R) also increase this susceptibility (Kwiatkowski, ٢٠٠٠; Bellamy, ٢٠٠٣; Rossouw *et. al.*, ٢٠٠٣). Genetic studies have revealed the presence of a gene named "natural- resistance- associated macrophage protein ١ (*Nramp* ١)" that controls resistance to the early growth of tubercle bacilli. This gene is located on chromosome ٢ and encodes a trans-membrane protein whose function is unknown, but whose DNA sequence has similarities to those of nitrate or iron transporters. Several polymorphisms in the *Nramp* ١ gene are associated with increased susceptibility to TB, and these polymorphisms are not found in whites, thus offering a possible explanation for the increased susceptibility of blacks to TB when

compared to whites. This susceptibility also is associated with abnormalities in HLA-DQ allele in humans (Bloom and Small, ٢٠٠٤).

١-٦ The aim of the study:

The aim of the present work was to evaluate the cellular immune functions in anergic tuberculous patients in comparison with allergic tuberculous patients and to normal subject.

To achieve this aim several steps were followed as in the followings:

١- Election of three human groups, group one anergic tuberculous patients (AN), group two allergic tuberculous patients (AL), and group three normal control subjects (C) .

٢- Affixing the character of BCG scar, and acid-fast bacilli (AFB) results for each group.

٣- Collecting blood samples with anticoagulant and sputa for each patient.

٤- Performing tuberculin delayed-hypersensitivity skin test for patients and controls.

٥- Making a differential leukocyte count for peripheral blood and sputa.

٦- Doing leukocyte migration inhibition, E-rosette, and migration inhibition, E-rosette, and nitrobluetetrazolium (NBT) reduction tests for patients and controls.

٧- In view of the results of the steps (١-٧), an explanation of anergy in pulmonary TB was made.

٨- Pointing out to a (test-battery) of choice for evaluation of cellular immune functions in tuberculous anergy.

٩- Putting down an operational or practical definition for anergy.

٢-١ The lungs:

The lungs are two spongy-like organs in the chest surrounded by a thin, moist membrane called the pleura. They are the largest organs in human's body. Each lung is composed of smooth, shiny lobes; the right lung has three lobes and the left has two. Approximately ٩٠٪ of the lung is filled with air and only ١٠٪ is soft-solid tissues. When any person inhales, the air travels through the following structures: Air is carried from the trachea into the lung through flexible airways called bronchi, which are divided successively into over a million smaller airways called bronchioles, which lead to a grape-like clusters of microscopic sacs called alveoli. In each lung of an adult there are millions of these tiny alveoli. Each alveolus has a thin membrane through which oxygen and carbon dioxide pass to and from capillaries, the smallest of blood vessels. During deep inhalation, the alveoli unfold and expand to allow fresh oxygen to pass into capillaries and remove carbon dioxide waste to pass out of the body through the lungs.The oxygen-rich blood is carried back through blood vessels to the heart, where it is pumped through the body (Franklin, ٢٠٠٤).

٢-١-١ The lung defense mechanisms:

Most large particles are removed from inspired air by the nose, which is composed of a stack of fine acrodynamic filters comprising fine hairs and columnar ciliated epithelium. The larynx act as a sphincter during cough and expectoration and is an essential mechanism protecting the lower airways during swallowing and vomiting (Hasellet *et. al.*, ٢٠٠٢). Beating of cilia of epithelial cells in the respiratory tract removes contaminating microorganisms that become trapped in the mucus. This

mechanism can be deranged by air pollutants, smoking, and alcoholism, all of which may predispose an individual to opportunistic respiratory infections (Hyde, ٢٠٠٠). Particles with a diameter greater than ٠.٥ µm that survive passage through the nose will be trapped by the lining fluid of the trachea and bronchi and cleaned by mucocilliary movement. In addition to its surface active properties of surfactant; a mixture of phospholipid, which are produced by a specific type of cuboidal epithelial cells (Type II-pneumocytes), Lung-lining fluids contain a number of proteins, including surfactant protein-A, which act as opsonins. It also contain other defense proteins including immunoglobulins (mainly S-IgA), complement, defensins, and a variety of anti-proteinases (including α_1 –antitrypsin), these proteins play a crucial role in protecting the lungs against invaders (Hasellet *et. al*., ٢٠٠٢).

Alveolar macrophages, which are one type of the cells of monocyte-macrophage lineage, normally patrol the interior of the alveoli, where they recognize and destroy bacteria and other foreign particles. They can also generate mediators, which cause an inflammatory response and attract granulocytes and monocytes, and present antigens. They exert importance scavenging functions in the clearance of dead bacteria and other cells during the aftermath of infection and inflammation (Hasellet *et. al.*, ٢٠٠٢).

٢-٢ Mycobacterial antigens:

Mycobacteria are complex unicellular organisms contain many antigenic proteins, lipids, and polysaccharides. Electrophoresis analysis of culture filtrates of *M. tuberculosis* reveals eleven precipitin arcs, which have been numbered and characterized. Antigens (١,٢, and ٣) are respectively, the polysaccharides arabinomannan, arabinogalactan, and glucan, and these components are common to all mycobacteria. Antigens ٦,٧ and ٨ are widely distributed proteins, while antigen ٥ is a glycoprotein with an antigenic epitopes apparently unique to *M. tuberculosis* (Grange, ١٩٨٨).

Tuberculin (purified protein derivative-PPD) is widely used to evaluate the cellular immune response to mycobacterial antigens. Koch's old tuberculin was a filtrate of a broth culture, which was then concentrated by evaporation in a heated water bath. This antigenic material contained various impurities derived from the medium and induced non-specific inflammatory reactions. To overcome this problem, Siebert in ١٩٣٤ attempted to isolate the tuberculoproteins which was termed as a purified protein derivative of tuberculin (PPD). The tuberculin antigens induce type IV delayed hypersensitivity (DTH) when introduced into immunologically active host (Grange, ١٩٨٨; Good and Shinnick, ١٩٩٨). An antigen of *M. tuberculosis* with an M.W of ٣٨,٠٠٠ D has been isolated by affinity chromatography using a monoclonal antibody, this ٣٨-kDa-antigen is present only in *M. tuberculosis* and *M. bovis*. It induces positive skin test reactions (Kadival *et. al.*, ١٩٨٧). The cellular immune response to the ٣٨ kDa-antigen is associated with current active disease, and is different from response to the other mycobacterial antigens like ١٤ kDa and ESAT-٦ antigens, which are associated with risk factors for

future active, but not current disease, suggesting that they might be useful to identify persons with higher risk of reactivation of latent TB (Silva *et. al.*, ٢٠٠٣). ESAT-٦, a T-cell antigen expressed in *M. tuberculosis* but absent in *M. bovis* BCG, may represent an alternative to PPD for skin test (Vincent *et. al.*, ٢٠٠٣). Immunization of mice with ESAT-٦ antigen induces protective immunity against TB, which is mediated by ESAT-٦ peptide-specific IFN-γ secreting CD_4 T-cells (Pathan *et. al.*, ٢٠٠١).

Phosphoantigens are nonpeptidic compounds with phosphoester structures, they are γ–derivatives of uracil-tri-phosphate (UTP) and deoxy-Thiamine-tri-phosphate (dTTP). These antigenic compounds are found only in *M. tuberculosis* and recognized by γδT-cells via TCR (T-cells receptors) (Rojas *et. al.*, ٢٠٠٢). The ٣٠ kDa antigen isolated from *M. tuberculosis* exerts sensitivity and specificity of skin test about ٧٢.٣٪ and ٩٠.٩٪ respectively, while in ٣٨ kDa antigen are ٧٣.٨٪ and ٨٤.٦٪ respectively (Selvaraj *et. al.*, ٢٠٠٣). *M. tuberculosis* ١٩ kDa antigen-reactive T-cells are elevated in acute pulmonary TB, declined with response to therapy, and resided in the terminally differentiated CD_8 T-cells subset (Hohn *et. al.*, ٢٠٠٣).

The mycobacterial surface is composed of many types of antigenic materials. The bacterium is enclosed within a typical lipid bilayer cytoplasmic membrane, which lies beneath rigid peptidoglycan (PG). A number of proteins are found in association with PG and between the membrane and PG, and some of these may be immunogenic, PG is covalently linked to arabinogalactan (AG). Mycolic acids are attached to the distal protein of AG. The complex of PG, AG, and mycolates of the disaccharide trehalose (cord factor) are also associated with the cell wall

skeleton (Fenton and Veremeulen, ١٩٩٦). The cord factor (Trehalose-٦, ٦́ –dimycolate) inhibits migration of leukocytes, causes chronic granulomas, and can serve as an immunologic adjuvant (Brooks *et. al.*, ٢٠٠٤). The abundant of glycolipids, lipids, and waxes are responsible for strong adjuvanticity, mycobacteria are the crucial components of Freund's complement adjuvant (Nossal, ٢٠٠٣).

٢-٣ Entry of Mycobacterial antigens:

The rout of entry of tubercle bacilli into the body is via the respiratory tract through the inhalation of respiratory droplets nuclei (Good and Shinnik, ١٩٩٨, Fenton and Vermeulen, ١٩٩٦). Droplets (١-٢) μm or less in diameter are inhaled into the lower respiratory tract. Droplets of a larger size are efficiently excluded from the lower respiratory tract by the physical barriers of the nasopharynx and upper respiratory tract (Schluger and Rom, ١٩٩٨). After inhalation, the droplet nucleus is carried down the bronchial tree and implants in a respiratory bronchiole or alveolus. Whether or not an inhaled tubercle bacilli establishes an infection in the lungs depends on both the bacterial virulence and the inherent microbicidal ability of the alveolar macrophage that ingests it (Dunlap *et. al.*, ٢٠٠٠). Bacteria that arrive in the deep lung are phagocytosed by alveolar macrophages (Goldsby *et. al.*, ٢٠٠٠). *M. tuberculosis* are thought to enter the macrophages via specific binding to several distinct cell surface molecules, and the precise route of pathogen entry is likely to determine the ultimate fate of bacilli within the macrophage, *M. tuberculosis* can bind directly via complement receptors and the macrophage mannose receptors (MMRc). The MMRc participates in nonopsonin-mediated phagocytosis by recognition of terminal mannose

residues on targeted particles (Fenton and Vermeulen, ١٩٩٦). Thus, mannose binding lectin facilitates phagocytosis of mycobacteria (Roitt *et. al.*, ٢٠٠١).

٢-٤ Antigen presentation of *M. tuberculosis*:

The immune system of humans and other mammals have evolved mechanisms for sampling the many proteins in their environments and cleaving them into peptides (antigen processing), and then making those peptides accessible for recognition by T-lymphocytes (antigen presentation). These processes are early, indispensable steps in nearly all acquired immune response to antigenic challenges (Brodsky, ٢٠٠١). The inhaled tubercle bacilli lodge in the periphery of the lung and are soon engulfed by alveolar macrophages, some bacilli are transported within macrophages to the local lymph nodes, when antigen is processed and presented to lymphocytes, which undergo clonal expansion (Grange, ١٩٨٨). Macrophages serve as important antigen presenting cells (APCs) in the host response to *M. tuberculosis*, likewise; dendritic cells can present mycobacterial antigens (Crevel *et. al.*, ٢٠٠٢; Fenton and Vermeulen *et al.*, ١٩٩٦). The mechanism of mycobacterial antigen presentation involves distinctive mechanisms, first major histocompatibility complex (MHC) class II molecules present mycobacterial proteins to antigen-specific $CD_{٤}$ T cell. These antigens must be processed in phagolysosome compartments in professional APCs. Second, MHC class I molecules, expressed on all nucleated cells, are able to present mycobacterial proteins to antigen-specific $CD_{٨}$ T-cells, this mechanism allows for the presentation of cytosolic antigens, which may be important as certain mycobacterial antigens may somehow escape the phagosome. Third, nopolymorphic

MHC class I molecules such as type I CD١ (a, b,and c) molecules, which are expressed on macrophages and dendritic cells, are able to present mycobacterial lipoproteins to CD١-restricted T-cells (Crevel *et. al.*, ٢٠٠٢). MHC-independent recognition of *M. tuberculosis* antigens may be characteristic of the response of γδT cells. Human γδT cells lines, which recognize mycobacterial antigens are derived from the peripheral blood of a healthy tuberculin responder (Haregewoin *et. al.*, ١٩٩١).These □δT cells accumulate in substantial numbers in mycobacterial lesions and exhibit strong reactivity toward mycobacterial antigens (Kabelitz *et. al.*, ١٩٩١). A small (٥٠٠-٦٠٠ Da) nonpeptide mycobacterial antigens isolated from *M. tuberculosis* can induce the proliferation of γδT cells in healthy subjects. This supports the hypothesis that some γδT cells can recognize nonpeptide antigens, macrophages processing and presentation of nonpeptide antigens (mycolic acids and lipoarabinomannan) is required for T-cell activation during TB infection (Fenton and Veremeulen, ١٩٩٦).

٢-٥ Macrophage functions during TB:

Once tubercle bacilli have made their way into the lung, they have four potential fates: (١) The initial host response can be completely effective and kill all bacilli, such that the patient has no chance of developing TB at any time in the future, (٢) The organisms can begin to multiply and grow immediately after infection, causing clinical disease known as primary TB, (٣) The bacilli may become dormant and never cause disease at all, such that the patient has what is referred to as latent infection manifest only by a positive tuberculin skin test, or (٤) The latent organisms can

eventually begin to grow, with resultant clinical disease, known as reactivation TB (Schluger and Rom, ١٩٩٨; DesJardin *et. al.*, ٢٠٠٢).

Macrophages are primary phagocytic cells in defense against *M. tuberculosis* (Fenton and Vermeulen, ١٩٩٦). Alveolar macrophages are capable of inhibiting growth of the bacillus through phagocytosis, and participating in a boarder context of cellular immunity through the process of antigen processing and presentation (٢.٤). Processes involved in phagocytosis include binding of the bacterium to the host cells, internalization, and finally growth inhibitor or killing. Binding of the organisms to the macrophages is carried out via complement receptor $CR_{١}$, $CR_{٣}$, and $CR_{٤}$, as well as mannose receptor (MR) (Schluger and Rom, ١٩٩٨). Leemans *et. al.*, (٢٠٠٣) have identified $CD_{٤٤}$ as a macrophage binding site for *M. tuberculosis* and mediates mycobacterial phagocytosis. Toll-like receptors (TLRs) are phylogenetically conserved mediators of innate immunity, which are essential for microbial recognition on macrophages and dendritic cells. TLRs are also involved in cellular recognition of *M. tuberculosis*. $MyD_{٨٨}$ (myeloid differentiation protein ٨٨), a common signaling component produced inside the macrophage, links all TLRs to IRAK (IL-١R-associated Kinase), which is a serine kinase that activates transcription factors like NF-KB to signal the production of cytokines. The $MyD_{٨٨}$ is also essential for *M. tuberculosis*-induced macrophage activation (Crevel *et. al.*, ٢٠٠٢).

Innate defense mechanism by phagocytic cells plays a key role in the eradication of TB infection, it has been found that acquired T-cell immunity in vaccinated mice protects them from disseminated TB, but does not prevent the initial pulmonary infection, in human disease, acquired T-cell immunity after vaccination with *M. bovis* BCG is more

effective against disseminated infection than against pulmonary TB. In racially integrated nursing homes, infection, as measured by tuberculin skin test, occurs twice as often in black as in white individuals who are equally exposed to active TB, this is due to the fact that innate host defense mechanisms are less efficient in blacks, and macrophages isolated from blacks are more relatively permissive for intracellular growth of virulent mycobacteria. These findings suggest associations between TB and functional gene polymorphism for various macrophage products, and indicate also the importance of macrophage in protective mechanisms against TB (Bellamy *et. al.*, ١٩٩٨; Bellamy *et. al.*, ١٩٩٩; Crevel *et. al.*, ٢٠٠٢).

Macrophages are the main effector cells involved in killing of *M. tuberculosis*, to become able to kill mycobacteria, macrophage, need to be activated. It is clear that lymphocyte product, mainly interferon-gamma (IFN-□), and proinflammatory cytokines like tumor necrosis factor-alpha (TNF-α) are important in macrophage activation (Schluger and Rom, ١٩٩٨; Wang *et. al.*, ١٩٩٩). Vitamine D seems involved in macrophage activation (Crevel *et. al.*, ٢٠٠٢). Two main mechanisms involved in killing *M. tuberculosis* within phagolysosome of activated macrophages:

١- The production of reactive oxygen intermediates (ROI) such as superoxide ($O_٢^-$) and hydrogen peroxide ($H_٢O_٢$), and

٢- Reactive nitrogen intermediates (RNI) by nitric oxide (NO) (Fenton and Vermeulen, ١٩٩٦; Crevel *et al.*, ٢٠٠٢).

One of the most effective mechanisms involved in macrophage defense against *M. tuberculosis* is apoptosis, or programmed cell death. This mechanism is mediated through a drown regulation of *bcl-*٢ gene, an inhibitor of apoptosis of macrophage results in reduced viability of

mycobacteria contained within it (Schluger and Rom, ١٩٩٨). *M. tuberculosis* are able to produce ammonia which serves to inhibit phagosome-lysosome fusion, and alkalinize the intralysosomal contents, and diminish the potency of the fusion complex. Similarly, sulfatides (derivatives of trehalose-٢-sulfate, and glycolipids) produced by *M. tuberculosis* is also able to inhibit phagosome-lysosome fusion (Schluger and Rom, ١٩٩٨). Evasion from the phagolysosome into the cytoplasmic compartments represents a highly successful mycobacterial survival, current evidences argue against perforation of the phagolysomal membrane by *M. tuberculosis* (Kaufmann, ٢٠٠٣). Infection of macrophage with *M. tuberculosis* can result in down regulation of MHC class II expression or presentation (Hmama *et. al.*, ١٩٩٨). *M. tuberculosis* also induces macrophages to produce immunosuppressive cytokines, such as interleukin-١٠ (IL-١٠) or TNF-β . These cytokines impair the ability of macrophage to stimulate T-cell effectivity (Hirsch *et. al.*, ١٩٩٤).

Stimulation of macrophages and dendritic cells with mycobacteria induce the production of TNF-α, which plays a key role in granuloma formation and activation of macrophages (Smith *et. al.*, ٢٠٠٢; Wang *et. al.*, ١٩٩٩). Without this cytokine, effective granuloma formation is diminished and bacterial numbers rapidly increase resulting in the increasing the severity of disease. The effects of TNF-α on the response to *M. tuberculosis* are multifaceted, and in addition to their action as macrophage activator, it enhances the RNI production and granuloma formation (Bean *et. al.*, ١٩٩٩).

M. tuberculosis has been shown to be readily phagocytosed by dendritic cells, but unlike, macrophages, accelerated intracellular growth is observed (Nagl *et. al.*, ٢٠٠٢).

Macrophages also produce transforming growth factor (TGF-β), which is widely distributed and produced mainly by monocytes and macrophages. It enhances macrophages chemotaxis and augmented expression of Fc receptors (Schluger and Rom, ١٩٩٨).

٢-٦ T-cells function during TB:

Elimination of *M. tuberculosis* infection depends mainly on the success of the interaction between infected macrophage and T-lymphocytes. $CD_{٤}^{+}$ T-cells exert their protective effect by the production of cytokines primarily IFN-γ after stimulating with mycobacterial antigens (Crevel *et. al.*, ٢٠٠٢). Yoneda and Ellner (١٩٩٨) stated that activation of *M. tuberculosis*-infected macrophage is mainly mediated by $CD^{٤+}$ T-cells and the phenomenon is MHC-restricted, $CD_{٤}^{-}$ T-cells are known to contain multiple subpopulations, including natural killer (NK) cells, $CD_{٨}^{+}$ T-cells and γδ T-cells. Functional diversity of T-lymphocytes may also be revelant, murine helper T (T_H) lymphocytes can be divided into two subsets: $T_{H١}$ clones, which are characterized by the production of IFN-γ, and $T_{H٢}$ clones, which are characterized by the production of IL-٤. IL-٢, produced by activated macrophages and dendritic cells, is the principal $Th_{١}$-inducing cytokine, while, IL-٤ promotes induction of $T_{H٢}$ cells (Abbas *et. al.*, ١٩٩٦). In mycobacterial infection, $T_{H١}$-type cytokine seem essential for protective immunity, IFN-γ gene polymorphism increase the susceptibility to *M. tuberculosis*, and individuals lacking receptors for IFN-γ suffer from recurrent, sometimes lethal mycobacterial infections (Crevel *et. al.*, ٢٠٠٢). The major role for IFN- in host defense is acting as

macrophage activator, and may also improve antigen presentation, leading to recruitment of CD^{4+} T-cells and/or cytotoxic T-cells, which may participate in mycobaterial killing (Schulger and Rom, ١٩٩٨). The quantitative assay of IFN-γ can be a useful tool in further studies of immune response to *M. tuberculosis* antigens and in the diagnosis of TB (Katial *et. al.*, ٢٠٠١; Mazurek *et. al.*, ٢٠٠١).

T_{H2}-type cytokines inhibit the *in vitro* production of IFN-γ, as well as activation of macrophage, and may therefore, weaken host defense. T_{H2}-type cytokines increases in TB patients (Crevel *et. al.*, ٢٠٠٢).

The revelance of the T_{H1}-T_{H2} concept in disease susceptibility or presentation remains uncertain (Crevel *et. al.*, ٢٠٠٢).

In addition to production of IFN-γ and other mediators, CD_4^+ T cells can function as class II MHC restricted cytotoxic cells, destroying monocytes/macrophages infected with *M. tuberculosis* or their products, ٦٥kDa heat shock protein expressed on mycobacteria stimulates CD_4^+T-cells to express cytotoxic activity against autologous macrophages (Yoneda and Ellner, ١٩٩٨).

CD_8^+ T-cells are likely to contribute as well, by secreting cytokines mainly IFN-γ and lysing infected cells (Crevel *et. al.*, ٢٠٠٢). CD_8^+ T-cells may be involved in cell lysis and apoptosis of infected cells, production of IL-٤, IFN-γ and thus may play a role in regulation the balance of T_{H1}-T_{H2} cells in the lungs of patients with pulmonary TB (Schulger and Rom, ١٩٩٨). Moreover, CD_8^+ T-cells contribute to the memory immunity by a combination of cytokines production and cytotoxicity (Serbina and Flynn, ٢٠٠١).

γ δ T-cells are non-MHC-restricted and hey function largely as cytotoxic T cells .The major role of γδ T-cells in human disease is their ability to act as cytotoxic for macrophages pulsed with mycobacerial antigens, and screte cytokines that may involved in granuloma formation, mainly TNFα (Schulger and Rom, ١٩٩٨). Also, γ δ T-cells may play an imporntant role by influencing local cellular traffic, promoting the influx of lymphocytes and monocytes (Dai *et. al*., ١٩٩٨). γ δ T-cells are induced by *M. bovis* BCG vaccination (Lee *et. al*., ٢٠٠٤).

٢-٧ B-cells functions during TB:

Although, B-cells play no direct role in immunity to intracellular bacterial infection like mycobacterial disease (Ryan and Projan, ٢٠٠١), the antibodies produced by plasma cells following TB infection have several roles: Amos *et. al*. (١٩٦٧) found that the sera of guinea pigs sensitized with BCG or tuberculin, contained cytophilic antibodies capable of fixing to macrophages and rendering them sensitive to migration inhibition by antigens. Stewart-Tull *et. al.* (١٩٧٥) showed that IgG produced during TB infection binds in its Fc portion to macrophage and by its Fab portion to antigen and thus can act as opsonin. IgE bind in its Fc to mast cells or basophils, and by its Fab to antigens. Perlmann *et. al.* (١٩٧٢) showed that the Fc portion of the antibody molecule is essential for cytotoxicity. The activation of cytotoxic cells, seem not to be T-cells, may come from interaction with antigen-antibody complexes on the surface of the target cells, these complexes are known to stimulate lymphocytes to increase DNA synthesis. Recently, antibodies against specific components of *M. tuberculosis* can be used in the diagnosis of TB, Florio *et. al.* (٢٠٠٣)

showed that IgG exerts antibody activity against *M. tuberculosis* isocitrate dehydrogenase, this immunoglobulin can be isolated from sera of TB patients.

٢-٨ Delayed type hypersensitivity induced by *M. tuberculosis*:

An immune response evokes a battery of effector molecules that acts to remove antigen. Generally, these effector molecules induce a sub clinical, localized inflammatory response that eliminates antigen without extensively damaging the host's tissue. Under certain circumstances, however, this response can have deleterious effects, resulting in significant tissue damage or even death, this inappropriate immune response is termed hypersensitivity or allergy (Goldsby *et. al.*, ٢٠٠٠). Coombs and Gell described four types of hypersensitivity reactions (types I, II, III, and VI), the first three types are antibody mediated; the fourth is mediated by T-cells and macrophages (Roitt *et. al.*, ٢٠٠١).

An individual immune to an intracellular pathogen will develop a skin reaction at the site of local administration of soluble antigens from this agent (Kaufmann, ٢٠٠٣).

The classic delayed type hypersensitivity (DTH) was first noted in ١٨٩٠, when Koch demonstrated that filtrates from cultures of *M. tuberculosis* could stimulate an inflammatory response, several hours after injection into animals (Eales, ١٩٩٩). Likewise, the reaction to intracutaneously injected tuberculin (PPD) is the typical example of a DTH (Dunlap *et. al.*, ٢٠٠٠).

The DTH as a reaction to antigens of *M. tuberculosis* is mediated by T-cells primarily of the $CD_{٤}$ phenotype. Because soluble protein antigens generally fail to enter the MHC class I pathway, contribution of convential $CD_{٨}$ T-cells to the DTH reaction must be considered low to absent.

Furthermore, an auxiliary role of γδ-T cells, however appears likely (Kaufmann, ٢٠٠٣).

Allergen penetrates the skin and is taken up by resident epidermal dendritic cells, also known as Langerhan's cells. Following capture of allergen, the dendritic cells differentiate into potent APCs and migrate to regional lymph nodes, there they encounter naive $CD_٤$ T-cells, triggering the activation of those T-cells whose antigen receptors are specific for the allergen in the form of processed antigen bound to the class II MHC molecules displayed on the surface of the APCs. The activated T-cells proliferate in the lymph node and differentiate into effector and memory cells, usually of the $T_{H١}$ type. When allergen again penetrates the skin, the memory cells rapidly evolve into effectors that mediate a DTH reaction at the site of penetration (Terr, ٢٠٠١).

As a mechanism of reaction, antigen-specific $CD_٤^+$ T-cells recognize the antigen, and secrete cytokines such as IFN-γ and TFN-α, which stimulate changes in the local tissue macrophages, causing them to be activated. Once these are stimulated in this way, they release toxic substances (ROI and RNI), which cause the destruction of both mycobacterial antigens and tissue cells. Moreover, Muramyl dipeptide, a component of mycobacterial cell wall, directly activates macrophages. This activation results in the production of a number of cytokines principally IL-١, which stimulate the secretion of IL-٢ and the expression of IL-٢ receptors on T-cells responding to antigenic stimulation. In addition, IL-١ is responsible directly or indirectly for many of the characteristic of DTH inflammation; for example, it causes fever by stimulating release of pyrogen from the hypothalamus of the brain and

synergies with TNF- α. The cytokines IFN-γ and TNF- α act on neutrophils, increasing their phagocytic activity by enhancement of superoxide anion production. These cytokines also enhance the antibody-dependent cellular cytotoxicity (ADCC) (Eales, ١٩٩٩). The lymphokines produced during DTH, induce induration through local vasodilatation, edema, and fibrin deposition (Colvin *et. al.*, ١٩٧٩). Features of tuberculin reaction, as an example of DTH reaction, include: (١) Its delayed course, reaching a peak more than ٢٤ h. after injection of the antigen; (٢) Its indurated character, and (٣) Its occasional vesiculation and necrosis.

Typically, the reaction of tuberculin begins ٥-٦ h. after injection, causes maximal induration at ٤٨-٧٢ h. and subsides over a period of days (Dunlap *et. al.*, ٢٠٠٠).

٢-٩ Immunological anergy associated with TB:

The interaction between an antigen and its specific cell receptors may result either in the activation and differentiation of the cell (positive selection) or in its inactivation or even death (negative selection). Positive selection usually results in a cell leaving the interphase (G٠) and entering the cell cycle, giving rise to a clone of increasingly differentiated effector cells, e.g., cytotoxic T-cells and plasma cells, this process is known as clonal selection. By contrast, when an antigen-receptor interaction results in negative selection leading to cell death, it is known as clonal deletion, although a single cell is destroyed, the host has lost the potential to develop the clone of cell which would recognize the epitope in question. The clonal selection is regarded as the effective mechanism to maintain the balance of self-non-self recognition. When the cell is not killed but made functionally inactive, the outcome is known as " *clonal anergy* "

(Eales, ١٩٩٩). Likewise, when T-cells recognize an antigen in a non-stimulating manner they become inactivated, producing a state of immunological tolerance, which is specific, only affecting T_H cells that respond to a particular antigen. Persistent tolerance without cell death is known as clonal anergy (Roitt *et. al.*, ٢٠٠١).

Anergy is defined clinically, as the absence or reduction of DTH skin test reactivity to commonly encountered antigens or it is a lessen reactivity of a previously positive DTH skin test, it may occur in individuals with extensive granulomatous diseases, such as miliary TB, severe coccidioidomycosis, leprosy, sarcoidosis, or Hodgkin's disease. A temporary loss of cell-mediated immunity (CMI) can occur during the acute phase of certain viral infections such as measles and in the various congenital forms of cellular immunodeficiency and in the acquired immunodeficiency syndrome-AIDS (Terr, ٢٠٠١). Anergy can be also defined as the absence of an expected immunological reactivity in sensitized individuals, it includes both the failure to express DTH (it is most common usage) as well as other types of immunological hyporeactivity including B-cell anergy (Hobart and McConnell, ١٩٧٥). The clinical conditions that induced impaired cell-mediated immunity like AIDS, malignancy, and immunosuppression therapy result in impaired class II-mediated DTH reaction (Cisneros and Murray, ١٩٩٦).

T-cells anergy has received a great deal of attention last years, several mechanisms were suggested to express the induction of this immune state. T-cell anergy is one mechanism thought to act in the periphery to ensure tolerance to self. The term anergy was first used to describe T-cells clones rendered unresponsiveness to subsequent re-stimulation by first activating them through the TCR (signal ١) without

appropriate co-stimulation signal (٢), anergic T-cells appeared to have a defect in signaling pathways upstream of transcription of the IL-٢ gene (Ermann *et. al.*, ٢٠٠١). In anergic cells, there is a discrete defect in the capacity of the TCR to couple to protein kinases, resulting in reduced the transcription factor-activating protein (AP-١) expression and transcription (University of Minnesota/Cancer center, ٢٠٠٢). AP-١ regulates the IL-٢ gene during T-cell activation, IL-٢ plays a central role in the activation of T-cells, therefore any defect in the AP-١ system may predisponse T-cells to be in anergic form (Kang *et. al.*, ١٩٩٢; Sundstedt and Dohlsten, ١٩٩٨; Oppenheim and Ruscetti, ٢٠٠١). The activation of T-cells directly depends on the interaction of $B_٧$, a co-stimulatory protein expressed on APCs, and $CD_{٢٨}$ expressed on T-cells, $CD_{٢٨}$ stimulation results in prolonging and augmenting the production of IL-٢, and other cytokines necessary for T-cells activation. T-cells may become anergic when the $B_٧$ engage T-cell associated CTLA-٤, which is a T-cell surface molecule induced on activation and not found on resting cells, instead of $CD_{٢٨}$. CTLA-٤ act as inhibitory receptor limiting T-cell activation (Avice *et. al.*, ٢٠٠١; Greenwald *et. al.*, ٢٠٠١; Roitt *et. al.*, ٢٠٠١). The mechanism leading to preferential interaction of $B_٧$ molecules with CTLA-٤ instead of $CD_{٢٨}$ remain to be determined (Avice *et. al.*, ٢٠٠١). Ermann *et. al.* (٢٠٠١) identified a gene related to anergy in lymphocytes (*GRAIL*) as a novel gene that is perferentially expressed in anergized T-cells, overexpression of *GRAIL* in T-cells dramatically reduced the transcription of IL-٢. The role of IL-٢ in the activation of T-cells can be supported by their use in the reversal of anergic T-cell into activated T-cells, the addition of exogenous IL-٢ to sub-optimally stimulated T-cells or to anergic T-cells drives

extensive T-cell proliferation and elicits reversal of clonal anergy (Beverly *et. al.*, ١٩٩٢).

During *in utero* life, CD٤٧ plays a key role in the induction of anergy, ligation of CD٤٧ in the absence of exogenous cytokine during primary activation of umbilical cord blood mononuclear cells (CBMCs) inhibits the expression of IL-٢ and induces a state of T-cell anergy (Avice *et. al.*, ٢٠٠١). Another cytokine that participates in the induction of anergy is IL-١٠, which induces anergy by down-regulating the expression of co-stimulatory molecules on the APC (Schols and Clercq, ١٩٩٦).

Anergy in the setting of TB refers to paradoxical absence of dermal activity to intradermal injection with PPD in infected persons. In anergic TB patients, sustained stimulation by *M. tuberculosis*, which results in IL-١٠ but not IFN-□ production, mediates the generation of anergic *M. tuberculosis*-specific T-cells with immunosuppressive properties. In the presence of IL-١٠, the *M. tuberculosis* infected host becomes tolerant to the *M. tuberculosis* antigens (Boussiotis *et. al.*, ٢٠٠٠). The development of *M. tuberculosis*-specific T-cell anergy due to chronic *M. tuberculosis* mediated stimulation in the absence of IFN-□ and the presence of IL-١٠ may contribute to the establishment of *M. tuberculosis* persistence, this persistence may hold the key to the problems of defective eradication of TB, resistance to treatment, and relapse (Bloom and McKinney, ١٩٩٩). There is far too much toxicity and tissue destruction in anergic TB patients (Grange, ١٩٨٨). The presence of immunosuppressive IL-١٠ producing T-cells in the peripheral blood of anergic TB patients suggest that M. *tuberculosis* mediates active inhibition of the host immune response, resulting in sustained survival of the infections organisms, thus induction

of anergy is regarded as one mechanism by which *M. tuberculosis* escapes the immune surveillance. For eradication of TB, besides chemotherapy, methods to reverse the *M. tuberculosis* induced anergy should be an integral part of novel treatment strategies attempting the cure and control of TB (Boussiotis *et. al.*, ٢٠٠٠).

٣-١ Study patients:

The samples consisted of one hundred and twenty five (٧٩ males and ٤٦ females) tuberculous patients of which ٨٢:١٢٥ were anergic, those whom giving negative tuberculin test. Meantime, ٤٣:١٢٥ were the allergic group which included those with positive turberculin test. The age range of pateints was (٦ months- ٨٠ years). These patients were admitted to the Babylon Center of Tuberculosis and Chest Diseases during the period July/٢٠٠٣ - May/٢٠٠٤.

Diagnosis of tuberculosis was established by the clinical picture, X-ray chest examination and positive sputum for acid-fast bacteria. Samples of blood and sputum were taken from each patient.

٣-٢ Study control (Normal subjects):

Twenty-three healthy subjects (١٨ males and ٥ females) were also studied as controls as clinical examination showed by respiratory clinician specialist. Blood samples were taken from each subject.

٣-٣ Buffers, Solutions and Media:

٣-٣-١ Physiological normal saline:

This solution was prepared according to (Cruickshank *et. al.*, ١٩٧٥).

٣-٣-٢ Phosphate Buffered Saline PBS (pH ٧.٢):

This buffer was prepared by dissolving one buffered disc in ١٠٠ ml distilled water and sterilized by autoclaving (١٢١C°, ١٥ pound/inch^{٢}, for ١٥ minutes) in accordance with the instructions of manufacturer (BDH).

٣-٣-٣ Alsever's solution (pH=٦.١):

To prepare this solution, the following ingredients were dissolved in ١٢٠٠ ml of distilled water:-

glucose	(BDH)	٢٤.٠٦	grams
Na-citrate	(BDH)	٩.٦	grams
NaCl	(BDH)	٥.٠٤	grams

The solution was sterilized by autoclaving (١٢١C°, ١٥ pound/inch^{٢}**,** for ١٠ minutes). This solution was used as anticoagulant, preservative and transport medium for sheep red blood corpuscles (Garvey *et. al.*, ١٩٧٧).

٣-٣-٤ Ficoll-Hyplaque (Lymphoprep):

This solution (Nycomed-Norway) was used for the isolation of lymphocytes. It was stored at ٤ C° in dark reagent bottles.

٣-٣-٥ Leukocyte-migration medium:

This medium was prepared by dissolving ١.٥-٢٪ agar A (MAST) in one liter distilled water, sterilized by autoclaving (١٢١C° ,١٥ pound/inch^{٢}**,** for ١٥ minutes) and poured into sterilized petri-dishes (١٠ cm. in diameter). This medium was used as a migration medium for leukocytes (Rose and Biggazzi, ١٩٨٢).

٣-٣-٦ Eagle medium:

This medium was prepared by dissolving one gram of Eagle medium (Sigma) in ١٠٠ ml distilled water and sterilized by membrane filtration (٠.٤٢ μm Whatman). It was used as nutritive medium for leukocytes in leukocyte migration inhibitory factor assay (Soborg, ١٩٦٩).

٣-٣-٧ Suspension of sheep red blood corpuscles (SRBCs):

This suspension was prepared as in the following:

Fresh blood was taken from neck vein of sheep and put into sterilized bottle containing Alsever's solution (٣-٣-٣) in a proportion volume: volume. The blood-Alsever's solution mixture was centrifuged at ٢٥٠٠ rpm for ١٥ minutes, and the sediment (red corpuscles) was washed three times with normal saline. Finally, the red corpuscles were re-suspended in Alsever's solution to a final concentration of red corpuscles (١٠٪), (Garvey *et. al.*, ١٩٧٧).

٣-٤ The stains:

٣-٤-١ Geimsa's stain:

Stock of Geimsa's stain, their buffer and fixative methanol were supplied by Vaccines and Sera Institute-IRAQ.

٣-٤-٢ Leishman's stain:

This stain was prepared in accordance with (Lewis *et. al.*, ٢٠٠١,a).

٣-٤-٣ Acid-Fast Stain:

The components of acid-fast stain (strong crabol fuchsin, H_2SO_4 ٢٠٪, and Loeffler's methylene blue) were supplied by Vaccines and Sera Institute-IRAQ.

٣-٥ Syringes:

٣-٥-١ Tuberculin syringes:

One milliliter graduated sterilized disposable plastic syringes –size G_{27} (Meheco/China) were used for injecting tuberculin into the skin of subjects.

٣-٥-٢ Blood collecting syringes:

Sterilized disposable plastic syringes –size G_{23} (Medical ject/Pakistan) were used for withdrawing the blood from subjects.

٣-٦ Pipettes:

Micropipette (١-١٠٠ µl – Slamed/Germany), glass pipettes with different sizes (٠.١, ٠.٢, ٠.٥, ١, ٢, ٥ and ١٠) ml and Pasteur's pipettes were used in this study.

٣-٧ Equipment and Tools:

The equipments used during this work were: -

The instrument	Company	Country
Autoclave	Stermite	Germany
Hot air oven	Memmert	Germany
Incubator	Memmert	Germany
Refrigerator	Concord	Lebanon
Balance	A&D	France
Centrifuge	Hermle Z ٣٢٠	Germany
Light microscope	Olympus	Japan
Microscopic optical reader	Medic	Italy
Haematocrit	Clay Adams	England
Cork borer	Locally manufactured	Iraq
Glass slides and coverslips	Meheco	Germany
Anticoagulant tube (K-EDTA)	AFMA-Dispo	Japan
Plain tube	AFMA-Dispo	Japan

٣-٨ Blood sampling:

Five ml of blood was withdrawn from an antecubital vein by syringes (٣-٥-٢). First, the skin was cleaned and made sterile by ٧٠٪ alcohol and allowed to dry before being punctured. Blood was slowly withdrawn by the piston of the syringe without an attempt to withdraw blood faster than the vein was filling. After the blood was delivered carefully from the syringe into anticoagulant tube and kept at ٤ Cْ until laboratory processing which was carried out without delay (Lewis *et. al.*, ٢٠٠١,a).

٣-٩ Sputum sampling:

Tuberculous patients were advised to wash their mouths three times with water. To obtain a sputum sample, the patient was given a labeled sputum container and was asked to :

- Inhale deeply from the chest.
- Open the container, bring it close to the mouth and bring the sputum out into it.
- Do not put saliva or nasal excretions into the container.
- Close the container.

Between two and five ml of sputum were collected from each patient. The type of sputum container was with a wide mouth, made of break-resistant plastic and with a screw cap to prevent leakage, desiccation and aerosol formation (Laszlo, ١٩٩٩; CDC, ١٩٩٥,a ; Grange, ١٩٨٨).The sputum samples were used for the demonstration of acid-fast bacilli and for direct examination of sputum to assess inflammatory process and the associated causative agents if any.

٣-١٠ Differential Leukocyte Count (DLC):

Blood film was made immediately as follows:-

After careful mixing of the blood, an appropriate drop was delivered by a capillary glass and placed in the center line of a clean microscope slide about one cm from one end. Then without delay, a spreader was placed in front of the drop at an angle of about ٣٠ْ to the slide and moved it back to make contact with the drop. The drop was spread out quickly along the line of contact. With a steady movement of the hand, the drop of blood was spread along the slide. The spreader was not lifted off until the last trace of blood has been spread out (Lewis *et. al.*, ٢٠٠١,a). After air drying, the blood film was stained by Leishaman's stain (٣-٤-٢):-

The slide was flooded with the Leishman's stain. After two minutes, double the volume of buffered water was added for ٥-٧ minutes. Then washed in a stream of buffered water until it acquired a pinkish tinge (up to two minutes), the back of the slide was wiped and set it upright to dry. Preparations were examined under oil immersion lens (١٠٠ X), counted ١٠٠ leukocytes and calculated the percentage of each type of leukocyte (Lewis *et. al.*, ٢٠٠١,a).

٣-١١ Examination of sputum:

٣-١١-١ Acid-Fast stain:

A- With a special care, sputum was homogenized for a few minutes with a clean woody stick.

B- A loopful from homogenized sputum was placed onto a clean microscopic slide and spread on the surface. The smear was dried in air.

C- The slide was covered with strong carbol fuchsin and heated until steam rose. Allow the preparation to stain for ٥ minutes, heat was applied at intervals to keep the stain hot.

D- The slide was washed with water.

E- Sulphuric acid (٢٠٪) was applied onto the slide for one minute. Then, the slide was washed with water.

F- The slide was flooded with counter stain Löffler's methylene blue for ١٠-٢٠ seconds.

G- The slide was washed, dried and microscopically examined at ١٠٠ X mycobacterium tuberculosis appeared as pinkish red bacilli, which loked as acid-fast positive bacilli (Cruickshank *et. Al.*, ١٩٧٥).

٣-١١-٢ Direct preparation:

Air-dried fixed sputum smear prepared as in the above was stained with Leishman's stain (٣-٤-٢) for the demonstration of the type of cellular infiltrate into the sputum.

٣-١٢ Immunoreagents:

٣-١٢-١ The tuberculin (PPD):

Tuberculin (purified protein drevative (PPD)-٢ units) preserved with phenol (٠.٥٪) was supplied by Vaccines and Sera Institute-IRAQ. It was used for the tuberculin skin test in patients and controls.

٣-١٣-٢ Nitroblue tetrazolium (NBT) dye:

This dye was prepared by dissolving ٠.٢ gram of nitroblue tetrazolium-NBT dye (٢, $\overline{٢}$-Di–p–Nitrophenyl–٥, $\overline{٥}$–diphenyl– 3, $\overline{3}$-(3, $\overline{3}$-Di-methoxy-4, $\overline{4}$-diphenylene) ditetrazolium chloride-Sigma $_{6876}$) in

100 ml of equal amount of sterilized normal saline (3- 3-1) and phosphate buffered saline –0.15 M (3- 3-2). It was stored in dark bottle at 4Cْ (Park *et.al,* ١٩٦٨). The NBT test was used as an assay method to screen the capacity of neutrophils to carry out oxidative metabolism of NBT in which NBT was reduced to formazan stippling as indicator for phagocytic capacity (Metcalf *et. al.,*١٩٨٦).

٣-١٤ Cellular Immune function tests:

٣-١٤-١ *In vivo* tests:

٣-١٤-١-١ Tuberculin (PPD) test:

٠.١ ml of tuberculin (٢ units) was administered with a ١ ml graduated syringe (٣- ٥-١). The injection was given intradermally in the middle third of the forearm. The skin was slightly stretched and the needlepoint inserted into the superficial layer of the dermis at angle ١٠-١٥ْ. The needle was being sensed as visible through the epidermis during insertion. The solution is slowly injected and a small papule of ٨-١٠ mm in diameter appeared, this meant that the solution was injected too deeply, in this case the test was repeated on the other arm. If the same arm was used, the injection site was changed to be separated at least ٤ cm from the first injection site. The result of tuberculin reaction was read ٧٢ hours after the injection (Statnes Serum Institute, ٢٠٠٢).

٣-١٤-١-٢ Bacilli-Calmette-Guiren (BCG) scare:

The scars of BCG vaccine were sought and recorded on the skin of subjects to determine the fashion of patient-vaccine interaction (Sousa *et. al.,* ١٩٩٧). Presence of scar indicated the positive interaction, whereas; absence of scare suggested the negative or abolished interaction.

٣-١٤-٢ *In vitro* tests:

٣-١٤-٢-١ Reduction of NBT dye:

٠.١ ml of fresh blood was mixed with ٠.١ ml. of NBT dye (١٣-٢), incubate at ٣٧ C̊ for ١٥ minutes and then at room temperature for an additional ١٥ minutes. At the end of this period, the blood-NBT mixture was mixed again. Blood film was prepared and stained with Leishman's stain (٣-١١). One hundred neutrophils were counted and only those neutrophils with a large black-blue deposits were classified as NBT positive (formazan stippling). The percentage of these neutrophils was recorded (Park *et.al.*, ١٩٦٨).

٣-١٤-٢-٢ Erythrocyte-Rosette Formation test (E-rosette test):

E-rosette means the clustering of sheep erythrocytes around a leukocyte or other cell. E-rosette formation is used as a marker for T-lymphocytes of humans and most mammals; in this case (E) are untreated, compared with other rosette tests such as EA where E have antibody (A) bound to their surface (Acor. Org., ٢٠٠٤).

E-rosette test is a method used to identify, isolate and quantitative the T-lymphocyte. This test was carried out according to (Burrell, ١٩٧٩; Gengozian *et. al.*, ٢٠٠٢; Frank, ١٩٩٧; and Madsen *et. al.*, ١٩٨٠):-

i- Three ml of Ficoll-Hypague (lymphoprep) was pipetted into a centrifuge tube.

ii- About ١.٥ - ٢.٠ ml of freshly drawn blood were carefully layered onto the surface of centrifuge tube without mixing.

iii- Centrifugation was carried out at ٤٠٠ g for ٣٠ minutes at room temperature.

iv- The leukocytes appeared as a fluffy white coating at the plasma-medium interface, while the erythrocytes appeared on the bottom of the tube, the upper plasma layer was carefully collected and saved it.

v- The white cells were collected with a Pastuer's pipette and placed it into another centrifuge tube.

vi- Centrifugation was performed at ٤٠٠ g for ٣٠ minutes and the supernate was discarded.

vii- The preparation was washed three times in PBS (٣-٣-٢) at ٤٠٠g for ١٥ minutes each.

viii- The pellet was re-suspended in ٠.٥ ml of saline to which ٠.٠٥-ml autologous plasma was added.

ix- To another centrifuge tube containing ٠.١ ml of plasma- lymphocyte mixture, ٠.١ ml of the sheep erythrocytes (٣-٢-٧) was added to the mixture and mixed gently.

x- The centrifuge tube was incubated at ٣٧ C° for ١٥ minutes, then centrifuged at ٢٠٠ g for ١٥ minutes.

xi- Incubation was carried out for ١ hour at ٤ C° with the supernate stilled on the pellet.

xii- The cells were re-suspended, very gently by tilting the tube back forth ٢-٣ times.

xiii- Blood film was prepared (٣-١١), dried in air and fixed with methanol for ١٠ minutes and stained with Geimsa's stain (٣-٤-١) for ١٠ minutes. The film was washed with buffered water, dried in air and microscopically examined under oil immersion lens (١٠٠ X).

xiv- One hundred lymphocytes were enumerated and the percentage of rosette-forming lymphocytes was recorded. Rosette-forming

lymphocytes were easily recognized by any lymphocytes with three or more sheep erythrocytes adhered to it.

٣-١٤-٢-٣ Leukocyte migration-Inhibitory Factor (LIF) test:

Leukocyte migration-inhibition factors mean protein factor(s) released by sensitized lymphocytes (and possibly other cells) that inhibit the movement of leukocytes, especially polymorphnuclear cells, away from their site of release. Assays for these factors are used as tests for cellular immunity (Medical dictionary online, ٢٠٠٤).

This test was performed in accordance with (Soborg, ١٩٦٩): -

i- By means of heparinized capillary tube, blood was drawn in duplicate for each patient and filling one end with plasticine.

ii- The blood capillaries were centrifuged in haematocrit for ٥ minutes.

iii- The capillaries were broken little above the buffy coat.

iv- These cutted capillaries were applied in ١٥ mm. agar-agar wells in petri-plates.

v- ٥٠ µl eagle medium and ٥٠ µl sensitizer antigen (tuberculin ٢ unite) were added into test wells and ٥٠ µl eagle and ٥٠ µl saline were added into a control well.

vi- In a humid jar, the plates were incubated in upright position overnight at ٣٧ C̊.

vii- Read the migration with and without sensitizer by using optical reader.

viii- LIF percent was calculated:

$$LIF \text{ percent} = \frac{Distance\ with\ sensitizer}{Distance\ without\ sensitizer} * ١٠٠$$

Inhibition more ٣٠٪ was significant.

٣.١٥ Statistical analysis:

Mean, median, and two tailed t-statistics were used as statistical parameters in this work (Cochran,١٩٧٤).

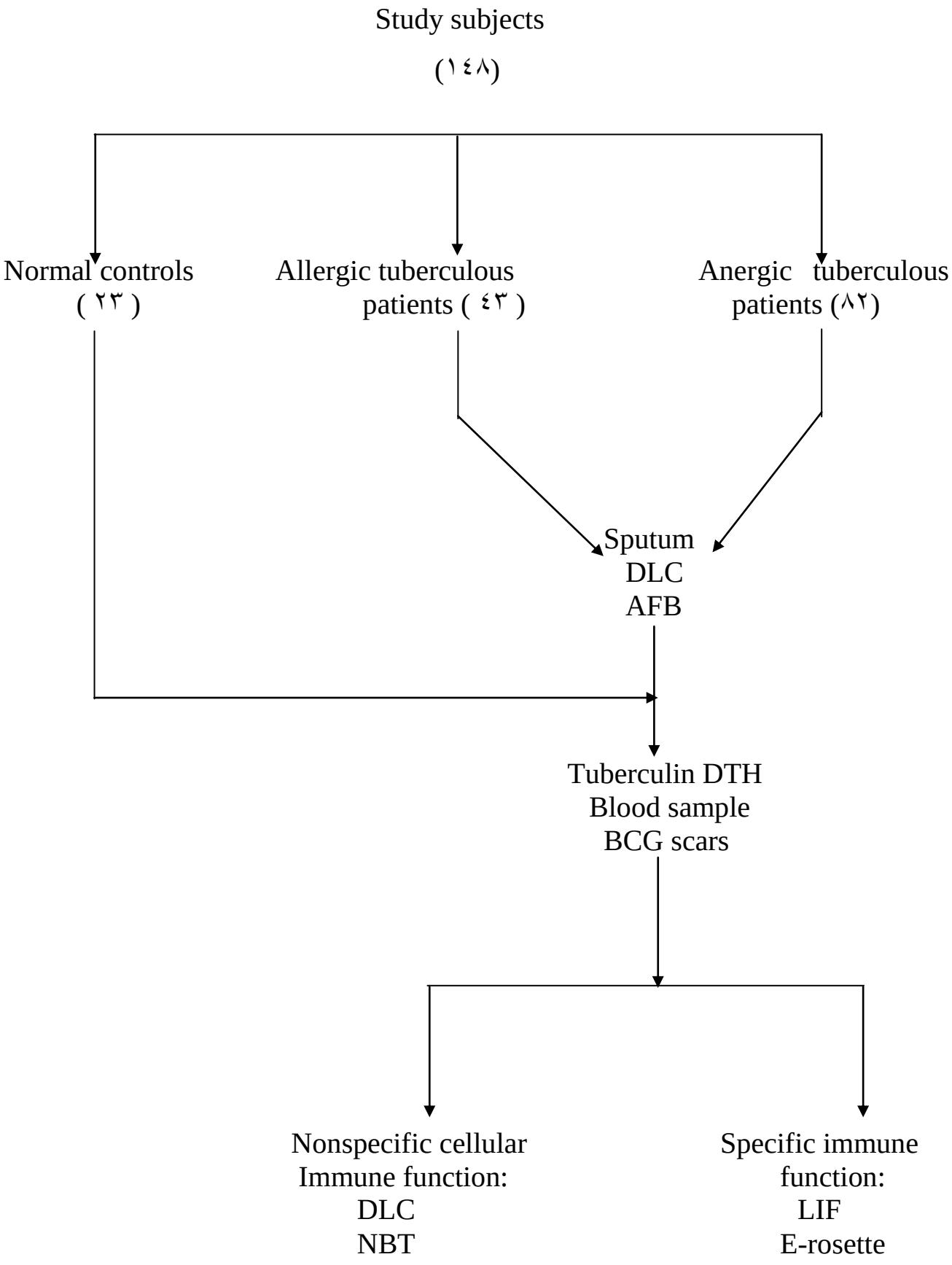

Figure ٣-١: Study flow chart.

٤-١ The study groups:

The study groups included anergic tuberculous patients (AN), allergic tuberculous patients (AL) and control subjects (C).

٤-٢ The test Battery:

The evaluation parameters followed to investigate the cellular immune state were *in vivo* and *in vitro* parameters. The *in vivo* parameters were of nonspecific as locally and systematically leukocyte responses, as well as the specific as tuberculin, BCG scars formation, and acid-fast stain. Meantime, the *in vitro* parameters were as nonspecific as NBT and specific as E-rosette, and LIF.

٤-٣ Score:

Results in tables (١-١٤) were expressed in a statistical manner in a sense of mean, and median. It was evident that the scientifically best of which representing the study case was the median. So, values throughout the text were expressed in this way. Except the occasions were the median value might be nil, mean values were scored.The in ter-group differences in the studied immune parameters were statistically evaluated using two tailed t-statistics and propability limit of (P=٠.٠٥)

٤-٤ Presentation:

The text fashion of the presented results was of comparative sense among anergic, allergic and controls groups. Tabulation and scattered diagram were mostly attempted. An inter-group classification of the study groups was also presented at certain

instances. Synopsis on the cellular immunology of anergic, allergic and control groups was made.

٤-٥ Age distribution:

Table (١) showed the age groups of the studied samples. AN exhibited an age range (٦ months-٨٠ years). The large age group ١٣:٨٢ (١٥.٨٢٪) was fitted with an age range (٢٥-٢٩) year. Likewise, AL expressed approximately the same age properties. Their ages were ranging from (١١-٧١) year and also, the large age group ٨:٤٣ (١٨.٦٠٤٪) was included in an age group (٢٥-٢٩) year. The third studied group, C showed an age range (١٨-٣٦) year and the large age group ١٠:٢٣ (٤٣.٤٧٨٪) was ranging from ٢٠ to ٢٤ years.

٤-٦ Sex influence:

The AN consisted of ٤٧:٨٢ (٥٧.٣١٧٪) males and ٣٥:٨٢ (٤٢.٦٨٣٪) females. AL consisted of ٣٢ out of ٤٣ males (٧٤.٤١٨٪) and ١١ out of ٤٣ (٢٥.٥٨٢٪) females. C was composed of ١٨:٢٣ (٧٨.٢٦٠) males and ٥:٢٣ (٢١.٧٤٠٪) females, table (١).

٤-٧ Leukocyte response:

٤-٧-١ Systemic leukocyte response:

The mean, and median of neutrophils were low, moderate and high in AN, AL and C. The median values were ٦١, ٥٥ and ٥٥٪ for C, AL and AN respectively. Lymphocytes medians were ٣٣, ٣٣ and ٣٧٪ for C, AL and AN accordingly.

Table (١): Age distribution and male-female ratio in AN, AL and C.

(Year)	AN	AL	C
٠.٥-<٥	٣	-	-
٥-٩	٤	-	-
١٠-١٤	٨	٢	-
١٥-١٩	٤	١	٤
٢٠-٢٤	١١	٧	١٠
٢٥-٢٩	١٣	٨	٢
٣٠-٣٤	٩	٨	٤
٣٥-٣٩	٢	٧	٣
٤٠-٤٤	٦	٢	-
٤٥-٤٩	٥	٣	-
٥٠-٥٤	٥	١	-
٥٥-٥٦	١	-	-
٦٠-٦٤	٤	٢	-
٦٥-٦٩	٣	-	-
٧٠-٧٤	٢	٢	-
٧٥-٧٩	١	-	-
٨٠	١	-	-
Age range (year)	٠.٥-٨٠	١١-٧١	١٨-٣٦
Total and male-female ratio	٨٢ (٤٧ male + ٣٥ female) ٥٧.٣١٧٪ male ٤٢.٦٨٣٪ female	٤٣ (٣٢ male + ١١ female) ٧٤.٤١٨٪ male ٢٥.٥٨٢٪ female	٢٣ (١٨ male + ٥ female) ٧٨.٢٦٠٪ male ٢١.٧٤٠٪ female

Monocytes median was similar in all three groups (٤٪). The eosinophils medians were ٢, ٤ and ٣٪ for C, AL and AN. Similarly, basophils medians were ٠, ١ and ٠٪ in C, AL and AL, table (٢).

٤-٧-٢ Local leukocyte response:

The neutrophils mean was higher in AN (١٢.٣١٥) than in AL (١٠.٥٢٥). The same observation was noted in lymphocytes (٧.٤٧٩) in AN and (٤.٣) in AL. Reversibly, monocytes mean in AL was higher (٠.٦٧٥) than in AN (٠.٣٢٨). Eosinophil means were approximately the same in AN (٠.٧٦٧) and (٠.٧٥٠) in AL. Basophils were completely absent in all sputum samples tested in AN. While the mean in AL was (٠.١), table (٣).

٤-٨ Eosinophilia:

The median of systemic eosinophilia was higher in AL(٤٪) than in AN (٣٪). Local eosinophilia exhibited nil value of median, but the mean of both AN and AL tend to be of similar value (٠.٧٦٧ and ٠.٧٥٠) respectively, table (٤).

٤-٩ Basophilia:

Systemic basophilia expressed a median (١٪) in AL, whereas, the median of basophilia in AN was nil. Local basophilia was absent in both types, but the mean of AL was (٠.١), in comparison to that of AN which was nil, table (٤).

Table (٢): Peripheral blood differential leukocyte count in tuberculous and control subjects.

Leukocyte type	AN		AL		C	
	Mean (%)	Median (%)	Mean (%)	Median (%)	Mean (%)	Median (%)
Neutrophil	٥٣.٧١٦	٥٥	٥٦.٤١٤	٥٥	٦١.١٧٠	٦١
Lymphocyte	٣٥.٦٣٥	٣٧	٣٢.٤١٤	٣٣	٣٢.٢١٧	٣٣
Monocyte	٤.٨٧٦٠	٤	٥.٤١٤٠	٤	٤.٠٤٠٠	٤
Eosinophil	٤.٧١٦٠	٣	٥.٤٣٩٠	٤	٢.٢٦٠٠	٢
Basophil	١.٠٠٠٠	٠	١.٠٢٤٠	١	٠.٣٠٤٠	٠

Table (٣): Leukocyte differential count of tuberculous patients in Sputum.

Leukocyte type	AN		AL	
	Mean (%)	Median (%)	Mean (%)	Median (%)
Neutrophil	١٢.٣١٥	١	١٠.٥٢٥	١
Lymphocyte	٧.٤٧٩٠	١	٤.٣٠٠٠	٠
Monocyte	٠.٣٢٨٠	٠	٠.٦٧٥٠	٠
Eosinophil	٠.٧٦٧٠	٠	٠.٧٥٠٠	٠
Basophil	٠.٠٠٠٠	٠	٠.١٠٠٠	٠

Table (٤): Local and systemic Eosinophilia and Basophilia in tuberculous patients.

Eosinophil	**AN**		**AL**	
	Mean	**Median**	**Mean**	**Median**
Sputum	٠.٧٦٧	٠	٠.٧٥٠	٠
Blood (%)	٤.٧١٦	٣	٥.٤٣٩	٤
Basophil	**Mean**	**Median**	**Mean**	**Median**
Sputum	٠	٠	٠.١٠٠	٠
Blood (%)	١.٠٠٠	٠	١.٠٢٤	١ ٠-٥

٤-١٠ Eosinophilia - Basophilia:

Table(٥) part-I revealed that in AL, peripheral blood leukocytes showed high mean of eosinophilia (٩.٥٪) and low for basophilia (١.٥٪). Meantime, AN appeared to be of two categories, category-١ exhibited low eosinophilia (٣٪) and high basophilia (٨٪), while, category-٢ was with high eosinophilia (٩.٣٪) and low basophilia (١.٥٪).

On comparing differential leukocyte count of blood and sputum for the same patient, it was evident, that AN and AL showed two basic categories. High eosinophilia in sputum and low in blood. Table (٥) part-II, showed that in AL, the mean of local eosinophil in category-١ was (٦.٥), while in systemic was (١.٥٪). The results in category-٢ were in contrast (١.٥) in local response and (٧.١٪) in systemic responses. Category-٣ represented the equivalence between two responses (٧٪) for each. In AN, category-١ showed a mean of eosinophil (٣.٢٥) and (٨.٥٪) for local and systemic responses respectively. Yet, these results inversely changed as in category-٢ (٦.٠) and (٢.٠٪). Meantime, these results tend to be similar to those obtained for basophil counting locally and systematically. In AL, category-١, showed absence of basophil in sputum and the mean percentage in blood was (١.٤٪). While in category-٢, the basophil means were (٢٪) for each type of responses. In AN, category-١ showed no basophil in each type of responses, in category-٢ local absence of basophil was recorded, but the mean percentage in systemic response was (٢٪).

Table (٥): Eosinophilia and Basophilia in Anergic and Allergic patients.

I-Blood		Eosinophils (E)			Basophils (B)		Conclusion
AL		٩.٥*			١.٥		High E Low B
AN	I	٣			٨		Low E High B
	II	٩.٣			١.٥		High E Low B
II- Sputum Blood		**Eosinophils**			**Basophils**		
		Sputum	**Blood**	**Conclusion**	**Sputum**	**Blood**	**Conclusion**
AL-I		٦.٥	١.٥	H.L**	٠	١.٤	L.H
AL-II		١.٥	٧.١	L.H	٢	٢.٠	E
AL-III		٧.٠	٧.٠	E	-	-	-
AN-I		٣.٢٥	٨.٥	L.H	٠	٠	-
AN-II		٦.٠٠	٢.٠	H.L	٠	٢	L.H

** L = Low , H = High , E = Equivalent.

* Mean.

٤-١١ The cellular immune function test:

٤-١١-١ NBT (Phagocytic activity test):

Figure (١-A) represented a photomicrograph of nitroblue tetrazolium (NBT) test (٣-١٣-٢). The reduction of soluble yellow dye into insoluble blue dark (formazan stippling) deposits indicated the positive reduction of NBT dye which reflected the phagocytic activity of neutrophils, while a negative results appeared as a clear cytoplasm without insoluble deposites due to inability of neutrophils to reduce NBT dye, figure (١-B).

The results of this test showed a gradual increasing values from AN, AL to C. The median value for AN was ٤.٠٪, for AL was higher than AN (٧.٠٪), and for C was higher than both two patients groups (١١.٠٪), table (٦).

Figure (٢) showed a scattered diagram of NBT test in AN, AL and C. this figure illustrated the gradual increasing of NBT values in AL and C in comparison to AN.

٤-١١-٢ E-rosette test (T-cell count):

Based on the fact that the T-lymphocytes were considered E^+-rosette forming T-cells when three or more sheep erythrocytes adhered to it, otherwise; they were regarded as E^--rosette forming T-cells. Figure (٣-A) showed a photomicrograph of E^+-rosette forming T-cells. While, figure (٣-B) showed E^--rosette forming T-cells.

As previously stated in NBT test, an increasing rate of E-rosette values was noted among AL and C on comparing with AN, likewise, this value was higher in C (٢٥٪) than AL (٢١.٠٪), while in AN was (١٤.٠٪), table (٧).

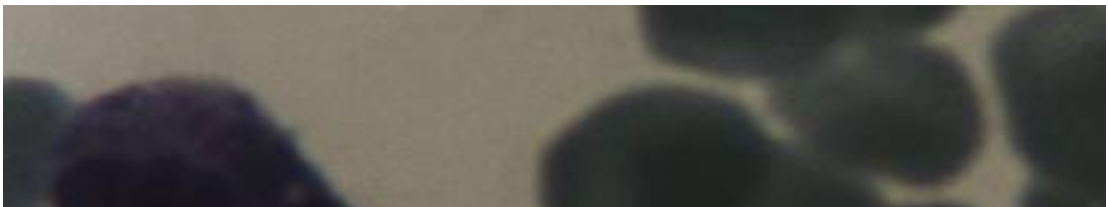

Table (٦): Nitroblue tetrazolium in tuberculous and normal subjects.

Patient groups	Median reduction values
AN	٤.٠٠
AL	٧.٠٠
C	١١.٠

Table (٧): E-rosette test for tuberculous and control subjects.

Patient groups	Median of E-rosette values (%)
AN	١٤.٠
AL	٢١.٠
C	٢٥.٠

Figure (٤) showed a scattered diagram of E-rosette in AN, AL and C. It was noted that the higher values were in C and to a lesser extent in AL , whereas the values tended to be relatively low in AN.

٤-١١-٣ Leukocyte Migration-Inhibition Factor (LIF):

LIF was estimated by using a capillary method (٣-١٤-٢-٣). Table (٨) revealed the results of LIF estimation in AN, AL and C. There were great differences in the LIF values among these three groups especially by comparing the LIF results of AL and C. Regarding the borderline of ٧٠٪, the LIF results of AL were significant (٣٧.٥٪), whereas the LIF results of AN were significant to a lesser extent (٥٧.١٤٪). But, it was clearly evident that the LIF results of C were non-significant (٨٨.٢٪).

Figure (٥) illustrated the scattered diagram of LIF values in all study groups. The group C was characterized by non significant values of LIF. While AL showed a significant values and AN exhibited less significant values.

Table (٨):Percent of Leukocyte- Inhibition Factor (LIF)* of peripheral blood in tuberculous and control subjects.

Patient groups	Median of LIF values (%)
AN	٥٧.١٤
AL	٣٧.٥٠
C	٨٨.٢٠

*The scoring of LIF was either as migration area or the mean of migration distance diameter; scores were following the second approach.

٤-١٢ Validation of cellular immunological parameters:

The main cellular immunological parameters attempted in this work were NBT, E-rosette and LIF. The results shown in table (٩) indicated the higher values for these three parameters in controls than tuberculous patients. Meantime, it was a degree of differences between AN and AL in the results of these parameters. NBT and E-rosette tests were higher in AL than AN and LIF tests showed more significantly values in AL when compared with AN.

E-rosette and LIF parameters, in general; revealed a great degree of differences among AN, AL and C, whereas NBT test showed; to a lesser extent, a degree of variation among these study groups.

The two tailed t-statistics to the inter-group differences of AL-C, AN-C, and AL-AN in cellular immune function tests were found to be significant in NBT for AN-C and E-rosette for AL-C (P=٠.٠٥)and highly significant for other cellular immune function tests (P=٠.٠٠١) table ٩-B. .Such statistical results are suggestive for their validity in use for evaluation of anergic state.

Table (٩-A): The evaluation of cellular parameters of tuberculous patients.

Patient groups	NBT-reduction (%)	E-rosette (%)	LIF (%)
	Median	Median	Median
AN	٤.٠٠	١٤.٠	٥٧.١٤
AL	٧.٠٠	٢١.٠	٣٧.٥٠
C	١١.٠	٢٥.٠	٨٨.٢٠

Table (٩-B): Two tailed t- statistics for the differences in cellular immune functions among the study groups.

Inter-group comparisons		Calculated $t_{٢٠+٢٠}$	Table t* value	P level	Significance
AL- C	NBT	٤.٨٠٥٠	٣.٩٢٢	٠.٠٠١	HS
	LIF	١٥.٢٤٥	٣.٩٢٢	٠.٠٠١	HS
	E- rosette	٢.٣٤٩٠	٢.١٠١	٠.٠٥٠	S
AN- C	NBT	٦.٩٤٣	٣.٩٢٢	٠.٠٠١	HS
	LIF	٤.٣٠٠	٣.٩٢٢	٠.٠٠١	HS
	E- rosette	٦.٨١٠	٣.٩٢٢	٠.٠٠١	HS
AN- AL	NBT	٢.٦٥٠	٢.١٠١	٠.٠٥٠	S
	LIF	٥.٤٤٩	٣.٩٢٢	٠.٠٠١	HS
	E- rosette	٥.٨٣٦	٣.٩٢٢	٠.٠٠١	HS

S= Significant HS= Highly significant * At df=١٨.

٤-١٣ The study patients, cellular Immunology:

٤-١٣-١ Control subject (C):

Table (١٠) showed the median value of age and the studied immunological parameters of C. These parameters were within the range of normal values.

٤-١٣-٢ Allergic Tuberculous Patients (AL):

The results of differential local leukocyte count revealed that the neutrophils were a main leukocyte type, followed by lymphocytes, eosinophils, monocytes and basophils. The last three types presented in a little value. Differential leukocyte count of peripheral blood showed elevated values for both eosinophils and basophils as compared to that of C and AN, table (١٠) and table (١١). NBT results were also within the normal range, but were less than C and at the same time they were greater than AN. E-rosette results gave a median value which was also considered as a normal value, but it was in its terminal normal range. Significant value was the characteristic feature of LIF assay, table (١١).

٤-١٣-٣ Anergic Tuberculous Patients (AN):

Table (١٢) showed the results of the studied parameters in AN. The differential leukocytes count of sputum revealed that the neutrophils and lymphocytes were the major leukocyte types. Median values of monocytes, eosinophils and basophils were nil. Differential leukocyte count of peripheral blood showed a degree of similarity between AN and AL, except in that of the median value of basophil was nil, while in AL was (١٪).

Table (١٠): Parameters of control subjects.

Statistical parameters	Differential leukocyte count (DLC) Blood* (%)	NBT (%)	E-rosette (%)	LIF (%)	Age (%)
Mean	Neutrophil (N): ٦١.١٧٠ Lymphocyte (L): ٣٢.٢١٧ Monocyte (M): ٤.٠٤٠٠ Eosinophil (E): ٢.٢٦٠٠ Basophil (B): ٠.٣٠٤٠	١١.٦٩٥	٢٥.٧٨٢	٨٥.٦٤٠	٢٥
Median	N: ٦١ L: ٣٣ M: ٤ E: ٢ B: ٠	١١.٠٠٠	٢٥.٠٠٠	٨٨.٢٠٠	٢٣

- There were no sputum samples.

Table(١١): Parameters of allergic tuberculous patients.

Statistical parameters	Differential leukocyte count (DLC)		NBT (%)	E-rosette (%)	LIF (%)	Age (%)
	Sputum (HPF)*	Blood (%)				
Mean	Neutrophil (N): ١٠.٥٢٥ Lymphocyte (L): ٤.٣ Monocyte (M): ٠.٦٧٥ Eosinophil (E): ٠.٧٥٠ Basophil (B): ٠.١	N: ٥٦.٤١٤ L: ٣٢.٤١٤ M: ٥.٤١٤٠ E: ٥.٤٣٩٠ B: ١.٠٢٤٠	٧.١١٤	٢٠.٧٩٠	٣٦.١٥٨	٣٣
Median	N: ١ L: ٠ M: ٠ E: ٠ B: ٠	N: ٥٥ L: ٣٣ M: ٤ E: ٤ B: ١	٧.٠٠٠	٢١.٠٠٠	٣٧.٥٠٠	٣٢

* HPF = High power field.

Table (١٢): Parameters of anergic tuberculous patients.

Statistical analysis	Differential leukocyte count (DLC)		NBT (%)	E-rosette (%)	LIF (%)	Age (%)
	Sputum (HPF)*	Blood (%)				
Mean	Neutrophil (N): ١٢.٣١٥ Lymphocyte(L): ٧.٤٧٩ Monocyte (M): ٠.٣٢٨ Eosinophil (E): ٠.٧٦٧ Basophil (B): ٠.٠٠٠	N: ٥٣.٧١٦ L: ٣٥.٦٣٥ M: ٤.٨٧٦٠ E: ٤.٧١٦٠ B: ١.٠٠٠٠	٥.٤١٢	١٤.٢٦٠	٥٢.٢٧	٣٢
Median	N: ١ L: ١ M: ٠ E: ٠ B: ٠	N: ٥٥ L: ٣٧ M: ٤ E: ٣ B: ٠	٤.٠٠٠	١٤.٠٠٠	٥٧.١٤	٢٨

*HPF= High power field

NBT results showed a great decreasing median value as compared to that of C and AL. Likewise, E-rosette results were less than C and AL. The last studied parameters, LIF; gave a moderate significant value which was less significant than AL.

٤-١٤ Diagnostic choice:

٤-١٤-١ BCG scars:

In AN, BCG scars were found in ٦١:٨٢ (٧٤.٣٩٥٪), in AL were high ٣٩:٤٣ (٩٠.٦٩٧٪), while in C were higher than both AN and AL ٢٢:٢٣ (٩٥.٦٥٢٪), table (١٣).

٤-١٤-٢ Tuberculin skin test:

The tuberculin skin test (٣-١٤-١-١) was negative in all AN , and depending on this test, patients were classified into AN (negative tuberculin skin test), figure (٦-A) and AL (positive tuberculin skin test), figure (٦-B), (Colombott and Benigni, ٢٠٠٢), while C were negative tuberculin skin test, table (١٣).

٤-١٤-٣ Acid-Fast stain:

Acid-fast stain (٣-٤-٣) showed that ١٥:٨٢ (١٨.٢٩٢٪) were positive (presence of Acid-Fast Bacilli (AFB)) in AN and ٥:٤٣ (١١.٦٢٧٪) in AL. This test was not performed in C due to the inability to obtain the sputum samples from healthy subjects, table (١٣).

Table (١٣): Comparative view to BCG scar, tuberculin and AFB positivity in tuberculous and control subjects.

Test groups	BCG	Tuberculin	AFB
AN	٦١:٨٢ (٧٤.٣٩٥ %)	٠ %	١٥:٨٢ (١٨.٢٩٢ %)
AL	٣٩:٤٣ (٩٠.٦٩٧ %)	١٠٠*	٥:٤٣ (١١.٦٢٧ %)
C	٢٢:٢٣ (٩٥.٦٥٢ %)	٠ %	-

*Test was positive if the diameter of induration was >=٥ mm

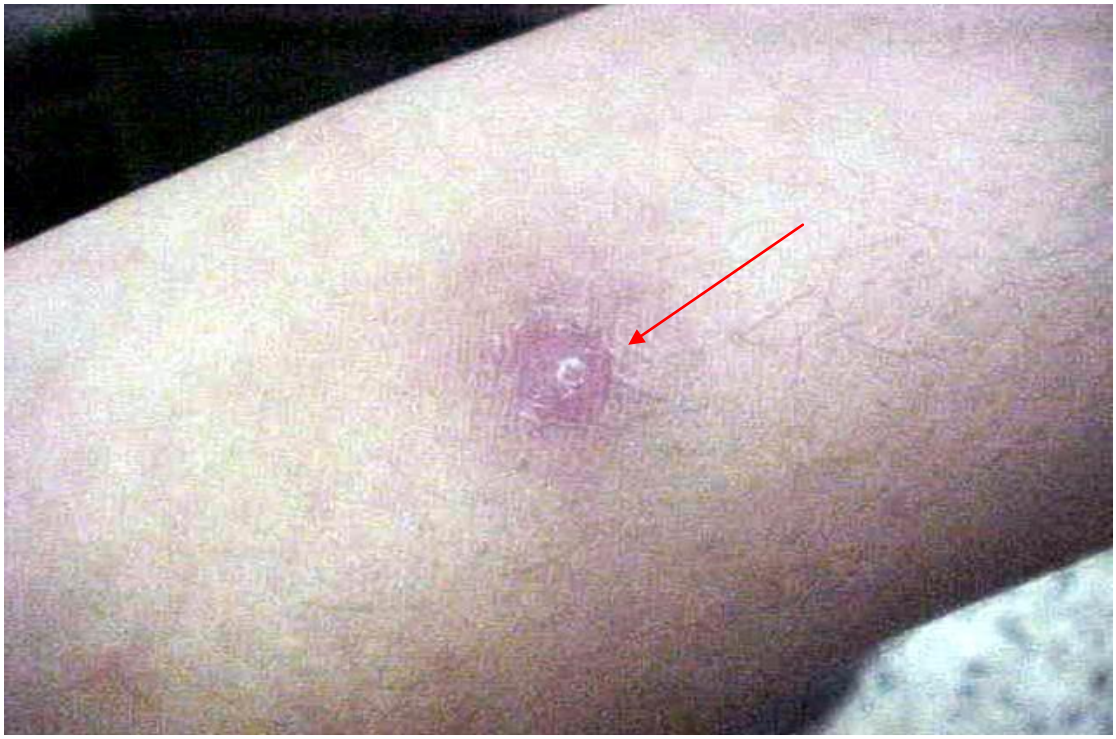

Figure (٦-A):Photomicrograph of Tuberculin skin positive test (→)

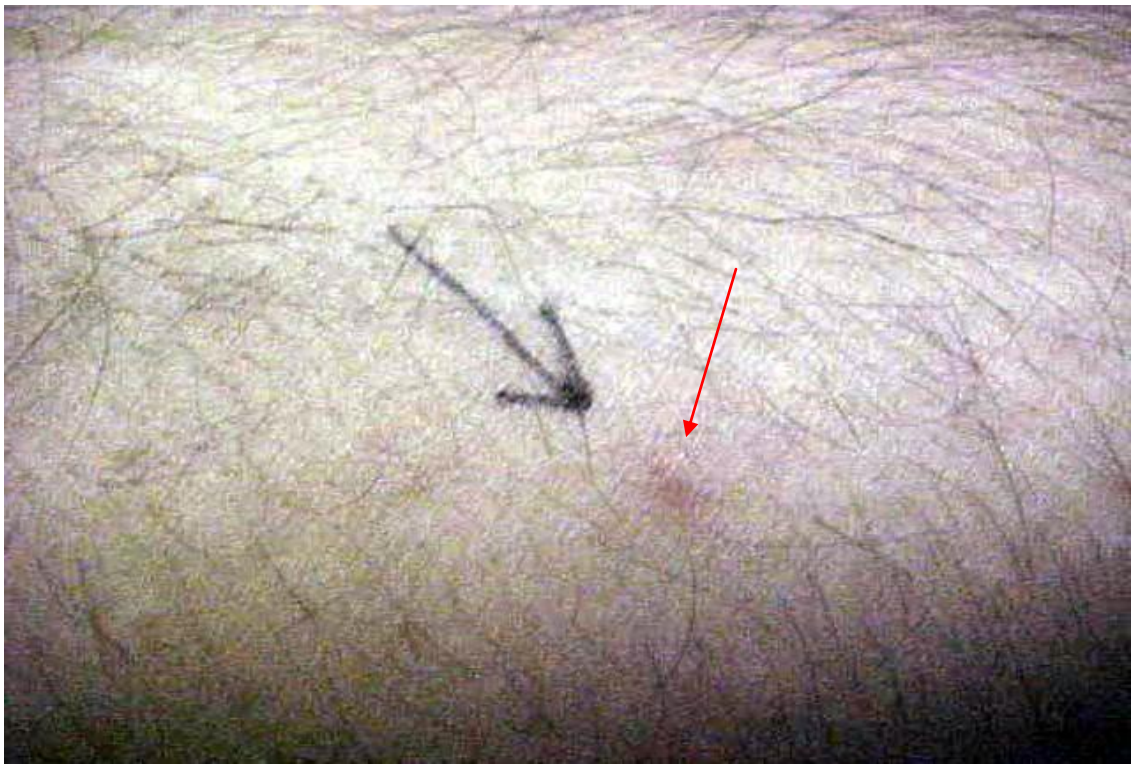

Figure (٦-B):Photomicrograph of Tuberculin skin negative test (→)

٤-١٤-٤ Specific and nonspecific parameters:

The nonspecific parameters used in this study were local and systemic differential leukocyte counts and NBT. These nonspecific parameters seemed to be general infection markers, table (١٤).

Collectively, the specific parameters were tuberculin, BCG scars formation, AFB, E-rosette and LIF. The last two parameters were showing a great difference among the studied groups. Such differences might give an indication for the anergic tuberculosis. Therefore; E-rosette and LIF represented clear probes in the identification and characterization of the immunological state of tuberculous patients. As diagnostic choice, E-rosette and LIF appeared to be more valid for the differential immunodiagnosis of tuberculous patients. This fact was more obvious when the results of these two parameters were compared among the study groups.

٤-١٥ Anergy, a practical or operational concept:

AN were with subnormal NBT ٥٧:٦٣ (٩٠.٤٧٦٪), subnormal E-rosette ٧٩:٨٢ (٩٦.٣٤١٪) and non-significant LIF ١:٧٨ (١.٢٨٪) as well as significant LIF ٧٧:٧٨ (٩٨.٧١٪). Meantime, normal NBT was in a ratio of ٢:٦٣ (٣.١٧٤٪), normal E-rosette was evident in a ratio of ٢:٨٢ (٢.٤٣٩٪). This anergy, can be real reduction in all or some of the cellular immune functions table (١٥). In comparison, subnormal NBT ٢٩:٣٥ (٨٢.٨٥٧٪), subnormal E-rosette ٣٨:٤٣ (٨٨.٧٣٢٪) and non-significant LIF was ٠:٤٣ (٠٪) and significant LIF ٤٣:٤٣ (١٠٠٪) in AL, table (١٦). It was evident that tuberculous infection, table (١٥) and table (١٦) exhibited in the host a sort of immunosuppression, but it was more evident in anergic patients.

Table (١٤): Specific and nonspecific cellular immune function in tuberculous patients and control.

Test groups	AFB (%)	BCG (%)	Tuberculin (%)	LIF (%)*	E-rosette (%)*	NBT (%)*
AN	١٨.٢٩٢	٧٤.٣٩٥	٠	٥٧.١٤	١٤.٠	٤.٠٠
AL	١١.٦٢٧	٩٠.٦٩٧	١٠٠	٣٧.٥٠	٢١.٠	٧.٠٠
C	-	٩٥.٦٥٢	٠	٨٨.٢٢	٢٥.٠	١١.٠

* Median value.

Table (١٥): Individual case assessment of anergy evaluation parameters.

		Mean	Median	Range
I) NBT	**C**	١١.٦٩٥	١١.٠	٦-٢٠
	AN	٥.٤١٢٠	٤.٠٠	١-١٤

Assessment as:	Subnormal	Normal	Abnormal
Ratio to AN:	٢٩/٦٣ (٤٦.٠٣١ %)	٦/٦٣ (٩.٥٢٣ %)	٢٨/٦٣ (٤٤.٤٤٪)
Ratio to C:	٥٧/٦٣ (٩٠.٤٧٦ %)	٢/٦٣ (٣.١٧٤ %)	٤/٦٣ (٦.٣٤٦ %)

		Mean	Median	Range
II) LIF	**C**	٨٥.٦٤٠	٨٨.٢٠	٧٨-٩٨
	AN	٥٢.٢٧٠	٥٧.١٤	٤.١-٩٠

Assessment as:	Subnormal	Normal	Abnormal
Ratio to AN:	١٢/٧٩ (١٥.١٨٩ %)	٤/٧٩ (٥.٠٦٣ %)	٦٢/٧٩ (٧٩.٤٨٧ %)
Ratio to C:	١/٧٨ (١.٢٨ %)	٠/٧٨	٧٧/٧٨ (٩٨.٧١ %)

		Mean	Median	Range
III)E-rosette	**C**	٢٥.٧٨٢	٢٥.٠	١٧-٣٦
	AN	١٤.٢٦٠	١٤.٠	٣-٢٦

Assessment as:	Subnormal	Normal	Abnormal
Ratio to AN:	٣٩/٨٣ (٤٧.٥٦٠ %)	٦/٨٢ (٧.٣١٧ %)	٣٧/٨٢ (٤٥.١٢١ %)
Ratio to C:	٧٩/٨٢ (٩٦.٣٤١ %)	٢/٨٢ (٢.٤٣٩ %)	١/٨٢ (١.٢١٩ %)

Table (١٦): Individual case assessment of allergy evaluation parameters.

		Mean	Median	Range
I) NBT	**C**	١١.٦٩٥	١١.٠	٦-٢٠
	AL	٧.١١٤٠	٧.٠٠	٢-١٤

Assessment as:	Subnormal	Normal	Abnormal
Ratio to AL:	١٧/٣٥ (٤٨.٥٧١ %)	٤/٣٥ (١١.٤٢٨ %)	١٤/٣٥ (٤٠ %)
Ratio to C:	٢٩/٣٥ (٨٢.٨٥٧ %)	٤/٣٥ (١١.٤٢٨ %)	٢/٣٥ (٥.٧١٤ %)

		Mean	Median	Range
II) LIF	**C**	٨٥.٦٤٠	٨٨.٢٠	٧٨-٩٨
	AL	٣٦.١٥٨	٣٧.٥٠	٤.١-٧٧

Assessment as:	Subnormal	Normal	Abnormal
Ratio to allergic:	١/٤٣ (٢.٣٢٥ %)	٠/٤٣ (٠ %)	٤٢/٤٣ (٩٧.٦٧٤ %)
Ratio to controls:	٠/٤٣ (٠ %)	٠/٤٣ (٠ %)	٤٣/٤٣ (١٠٠ %)

		Mean	Median	Range
III)E-rosette	**C**	٢٥.٧٨٢	٢٥.٠	١٧-٣٦
	AL	٢٠.٧٩٠	٢١.٠	١١-٢٦

Assessment as:	Subnormal	Normal	Abnormal
Ratio to AL:	١٨/٤٣ (٤١.٨٦٠ %)	٥/٤٣ (١١.٦٢٧ %)	٢٠/٤٣ (٤.٦٥١ %)
Ratio to C:	٣٨/٤٣ (٨٨.٣٧٢ %)	٣/٤٣ (٦.٩٧٦ %)	٢/٤٣ (٤.٦٥١ %)

٤-١٦ Normal values:

Table (١٧) showed the normal values of parameters used in this work. There were two recorded normal values, one represented that recorded by literatures and another recorded by this work.

Table (١٧): Normal values of parameters used in this study.

Parameters	Normal values		
	In litretures	References	In this work
• Differential leukocyte count of sputum	Nil	Ravel, ١٩٨٤	-
• Differential leukocyte count of peripheral blood - Neutrophils - Lymphocytes - Monocytes - Eosinophils - Basophils	- ٤٠-٧٥ % ٢٠-٤٠ % ٢-١٠ % ١-٥ % < ١ %	Mason, ٢٠٠٤	- ٦١ % ٣٣ % ٤ % ٢ % ٠ %
• E-rosette	٢١-٣٣ %	Burrell, ١٩٧٩	٢٥ %
• NBT	٨.٥ %	Park *et. al.*, ١٩٦٨	١١ %
• LIF	Border line ٧٠ %	Soborg,١٩٦٨	- Border line ٧٠ %
• Tuberculin	≥ ٥ mm.	CDC,١٩٩٦,a	≥ ٥ mm

٥-١ Age influence:

The results that were expressed in table (١) revealed the wide age ranges in both patient groups. This suggests that all age groups were susceptible to TB infection. This was in agreement with what have been reported by (Kaltenbach *et. al.*, ٢٠٠١) who showed that there is no significant difference in the infection with *M. tuberculosis* between young and elderly patients.

The TB infection involves all age groups, but the rate of infection varies depending on the age, ٨٠٪ of all TB cases are in people more than ٥٠ years in industrialized countries. It was estimated that about ٧٥٪ of all TB cases were in people less than ٥٠ years in developing countries and ١,٣٠٠,٠٠٠ TB cases were recorded in age less than ١٥ years with a mortality rate about ٣٣٪ (Layne, ٢٠٠٣). Haselett *et. al.*(٢٠٠٢) revealed that one-third of TB cases occurs in young adults. The extreme points of age ranges play an important role in the determination of clinical manifestation of TB, in newborns and elderly; the tuberculin skin test are mostly false negative (Dunlap *et. al.*, ٢٠٠٠). In developed countries a substantial increase in the notification rate amongst elderly people has been observed in case of TB. This increase may be attributed to the lowered resistance and health ignorance at the age range termini (Al-Damluji, ١٩٧٦). Locally, (Al-Damluji, ١٩٧٦) revealed that the most cases of TB in Iraq were in people less than ١٥ years.

٥-٢ Sex influence:

Male-female ratio stated in table (١) showed that the rate of TB, in both patient groups; was higher in males than in females. This finding was matched with that recorded by (Al-Damluji, ١٩٧٦) who mentioned that males are more susceptible to TB infection than females. This susceptibility may be attributed mainly to immunosuppressive effect of alcoholism, smoking, more occupational exposure and severe stress which are considered predisposing factors for the infection with *M. tuberculosis* (Al-Damluji, ١٩٧٦; Layne, ٢٠٠٣).

Leukocyte responses:

٥-٣-١ Systemic leukocyte response:

Results in table (٢) showed a relative lower values of neutrophils in differential leukocyte count stained films of tuberculous patients (AN and AL) in comparison to that of controls. This relative decreasing value of neutrophils may be attributed to long-term drug treatment (Haselett *et. al.*, ٢٠٠٢) and ability of *M. tuberculosis* to induced apoptosis in peripheral blood neutrophils in patients with active tuberculosis (Aleman *et. al.*, ٢٠٠٢). Lymphocyte showed a relative increasing value in AN when compared to AL and C which were approximately of the same values. Chronic infections, like TB cause an increasing in lymphocytes (Bradlow, ٢٠٠١; Child and Cuthbert, ١٩٩٢). Eosinophils showed a relatively increase value in tuberculous patients (AN and AL) when compared to that of controls. This result was fitted to that reported by (Dunlap *et. al.*, ٢٠٠٠). Monocyte median count values were similar in all studying groups,

while (Haselett *et. al.*, ٢٠٠٢) and (Child and Cuthbert, ١٩٩٢) stated that TB is considered as one of the causes of monocytosis. Basophils showed a degree of similarity among the three studying groups. Although, there is an indication that one of the common causes of basophilia is TB (Schick and Austen, ١٩٨٧).

٥-٣-٢ Local Immune Response:

The results expressed in table (٣) revealed that the neutrophils was the predominant leukocyte type in both patient groups, followed by Lymphocyte. Likewise, the mean and range of Lymphocytes were higher in AN than in AL in contrast with that of monocytes which were higher in AL than in AN.

The studies on bronchoalveolar lavage of tuberculous patients show that the inflammatory responses increase in parallel with the severity of disease and the pattern of inflammation in bronchoalveolar lavage fluid is not similar to that in peripheral blood, particularly in patients with advanced lesions (Dhand *et. al.*, ١٩٨٨). The separation in the pattern of local and systemic responses tended to mimick the nature of mucosal immunity which are regarded as independent mechanism (Brandtzaeg, ١٩٩٦). Condons *et. al.* (١٩٩٨) revealed that there is a great degree of correlation between the type of local cellular immune response in tuberculosis with particular manifestations of the disease. In patients with sputum smear negative AFB and no cavities on chest radiograph, the lymphocytes will be the predominant local cells, whereas in patients with sputum smear positive AFB, cavitation and advanced pulmonary tuberculosis on chest radiograph, neutrophils will be the local predominant cell type. On the other hand, patients with less frequency of smear positive AFB or cavitation, are characterized by the

predominance of alveolar macrophages in their bronchoalveolar lavage. Ainslie *et. al.* (١٩٩٢) reported that lymphocyte numbers in bronchoalveolar lavage fluid vary widely in localized pulmonary and miliary tuberculosis but are highest in lavage fluid in patients with miliary tuberculosis. They attributed this to an increase in CD_8 lymphocytes. (Ozaki *et. al.*, ١٩٩٢) have illustrated that both neutrophils and lymphocytes were increased in number in bronchoalveolar lavage fluid from affected lesions of the lungs of patients with miliary tuberculosis and patients with active pulmonary tuberculosis compared with those in bronchoalveolar lavage fluid in control subjects, but the number of alveolar macrophages was decreased in bronchoalveolar fluid from tuberculous lesions. Hoheisel *et. al.* (١٩٩٤) showed that the proportion of lymphocytes was increased in bronchoalveolar lavage fluid in pulmonary tuberculosis.

The growth of *M. tuberculosis* seems to depend in large part on the interaction between T-cells and macrophages. A good deal of attention has focused on the role of IFN-ɣ secreted locally and its ability to activate macrophages to inhibit mycobacterial growth. Onwubalili *et. al.* (١٩٨٥) mentioned that this cytokine is acting primarily as macrophage activator and improves antigen presentation leading to recruitment of CD_4^+ T-cells and cytotoxic T-cells which may participate in mycobacterial killing. In patients without alveolar lymphocytosis, levels of IFN-ɣ were low, but these levels increased on antituberculous therapy as the patients improve which correlates directly with an increasing in CD_4 T-cells count, as the major producer of this cytokine. (Kaplan *et. al.*, ٢٠٠٣) revealed that failure to control tubercle bacilli in tuberculous patients is not associated with a

generalized suppression of cellular immunity, but is mainly due to a selective absence of $CD_{٤}^{+}$ and $CD_{٨}^{+}$ T-cells at the luminal surface of lung cavity.

Neutrophilic alveolitis is associated with more advanced disease and there is a relationship between *M. tuberculosis* and IL-٨, a cytokine that is a potent neutrophilic attractant; the cellular components of *M. tuberculosis* were capable of stimulating both gene expression and protein secretion of IL-٨ from alveolar macrophages (Zhang *et. al.*, ١٩٩٥). Neutrophils is mainly associated with severe pulmonary disease and represent a harmful local response resulting in local tissue damage (Condons *et. al.*, ١٩٩٨).

Sex and age have no influence on the pattern of local immune response to pulmonary tuberculosis (Dhand *et. al.*, ١٩٨٨). The type of local immune response in the lung is linked to a large extent with the clinical presentations and outcomes. Understanding the precise mediators of this response may lead to new insights into the pathogenesis, manifestation and treatment of tuberculosis (Condons *et. al.*, ١٩٩٨).

٥-٣-٣ Eosinophilia:

The first description of eosinophil leukocyte was at ١٨٤٦. in which it had been described as “coarse granular cells” in vertebrate blood including man. Recognition of the cell as distinct entity was established in ١٨٦٥, but the term “ eosinophil“ was first used by Erlich in ١٨٧٩ ; as a result of his observation that the granular had an affinity for acid aniline dyes (Kay, ١٩٨٤). The colorful appearance of eosinophils in Romanofsky-stained blood smears has led to vast amount of literature concerning them, but it is only in the past two

decades that their role in host defense is clarified (Boggs and Winkelstein, ١٩٨٦).

The overall configuration and the configuration of bone marrow precursor compartments for both eosinophils and neutrophils are very similar, but the two types have distinct terminal stem cells as well as distinctively different chemistries, kinetics and functions. Colonies of eosinophils can be stimulated to grow independently of colonies of neutrophils–macrophages by the action of a specific colony stimulating factors (CSF). This CSF is produced by activated T-lymphocytes and perhaps by other cells and there is *in vivo* evidence that eosinophil production increases in helminthic infection, bacterial infections and post-bacterial infections and this increasing may be mediated by T-lymphocytes. Eosinophils can respond to certain *in vivo* inflammatory chemotactic stimuli. Many eosinophils are normally found just below epithelial surface where these are exposed or potentially exposed to external environment, such as skin, lungs, gut, lower respiratory tract and uterus (Boggs and Winkelstein, ١٩٨٦). So, they can ingest antigen-antibody complexes and attack helminths as well as contribute in histamine release (Lessof., ١٩٨٧).

The eosinophils overall pattern of marrow production, adhesion, migration, attachment, phagocytosis and killing were as that outlined in literatures for neutrophils. But eosinophils; generally, are sluggish and inefficient in phagocytosis and killing of bacteria (Boggs and Winkelstein, ١٩٨٦). The blood transit-time of eosinophils is approximately twice as long as that of neutrophils and unlike neutrophils, the eosinophil probably returns from tissue to blood and from blood to marrow in normal conditions. Under pathophysiologic or pathologic conditions eosinophils have either notable increase or

notable decrease in their absolute or differential count percentages. The first case however, can be stated as "eosinophilia" and the second as "eosinopenia" (Nicholson, ١٩٨٤).

Eosinophilia state can be attributed to several causes among which are: parasitic infections, bacterial infections and allergic conditions (Nicholson, ١٩٨٤). Much more common than eosinophilia is the eosinopenia; the absence of eosinophils on repeated examination of blood, marrow and other tissues is abnormal and indicative for eosinopenia. Eosinopenia are noted in several clinical situations like: allergy, steroid therapy, stress and bacterial infections. The aetiology of eosinopenia is multifactorial such as loss of blood eosinophils to the inflamed area and cessation of their release from marrow (Boggs and Wilkelstein, ١٩٨٦). Mucosal as well as systemic eosinophilia were noted both among anergic and allergic pulmonary tuberculosis (tables (٤), (٥)). Patients were with systemic others were with mucosal and a third group showing both at peripheral blood and mucosal levels (Chandran *et. al.*, ١٩٨٥; Chan *et. al.*, ١٩٨٨; Hsu *et. al.*, ٢٠٠٠) some workers implicated the occurrence of mucosal eosinophilia to systemic IL-١٥ production (Wang *et. al.*, ١٩٩٨). In a murine model; however, it was demonstrated that although eosinophilia is not a feature of $T_{H١}$ predominance, DTH reactions, these reaction produced chemoattractants for eosinophil migration and the control of circulating eosinophil level appear to be most important strategy in determining tissue eosinophilia (Teixeira *et. al.*, ٢٠٠١).

٥-٣-٤ Basophilia:

The origin of basophils is not known. It is morphologically similar to mast cells. it is also not known whether these two cell types

have a common origin. There are clear morphological and biochemical differences have been described for the two types. Basophil leukocyte are often presumed to originate from stem cells in bone marrow (Foreman, ١٩٨٤). Basophil granules are that appropriately termed as "suicide bags" with segmented nucleus. Basophils are produced in marrow via the same general manner outlined for neutrophils (Boggs and Winkelstein, ١٩٨٦). Basophils have high affinity membrane receptors for the Fc portion of the IgE molecules. They are found in circulating system a variety of immunologic and non immunologic stimuli can initiate basophil exocytosis with releasing of secretory granules components which include biologic amines, proteoglycans, neutral proteases, acid hydrolyses and arachidonic acid metabolites. Basophils are classified by the content of their secretory granules, location, morphology, staining characteristics and T lymphocyte dependence. Although basically, basophils are generally located in the blood, but it can occasionally infiltrate tissues. While, apparently, there are no evidence for the classification of basophil as connective and mucosal basophils as that of mast cells (Schick and Austen, ١٩٨٧).

Basophilia is the increase of basophils count in peripheral blood to more than ٥٠ cell per $mm^{٣}$ and is associated with hypersensitivity reactions, myeloproliferative disorders, infections disorders, gastrointestinal disorders and haematologic disorders. Among the infections disorders, the chronic infectious disease "tuberculosis" (Bielory and Gascon, ١٩٨٧). Basophils are shown to have IgE surface receptors of glycoproteins natures. The polyvalent antigens induce the basophil to release histamine and other pharmacologically active substances. Through IgE cross-linking on their surface (Foreman, ١٩٨٤). Basophils count in peripheral blood was increased in relation to

asthma, associated symptoms and to airway hyper reactivity and increased IgE levels but differed from eosinophils in that basophils were related to atopy and the actual role of basophil in human lung disease is most clearly undescribed (Lewis *et. al.*, ٢٠٠١, b). The proove of mucosal and/or circulating basophilia (tables (٤), (٥)) came in agreement with that mentioned by (Bielory and Gascan, ١٩٨٧) in which tuberculosis constitutes one of the main causes of basophilia. Meantime, it was flowing with finding that human lung disease can be associated with basophilia (Lewis *et. al.*, ٢٠٠١, b). Actually, variable grades of eosinophilia vs. basophilia were noted among anergic and allergic tuberculous patients with different profiles of circulating and tissue basophilia or eosinophilia.

Basophilia is related to atopy while Eosinophilia is unrelated to atopy (Lewis *et. al.*, ٢٠٠١). However; basophilia is evident in tuberculosis (Bielory and Gascan, ١٩٨٧).

٥-٣ The cellular immune function tests:

٥-٤-١ NBT (Phagocytic activity test):

NBT is a test that measures the ability of certain cells (neutrophils) in the immune system to reduce the colorless chemical, nitrobluetetrazolium (NBT), to a deep blue color (Milone ,٢٠٠٣). The mechanism of nitrobluetetrazolium test depends on the NADPH oxidase (class I: oxidoreductase), which is responsible for the respiratory burst and the generation of superoxide. The NADPH oxidase is a pentameric-protein complex that in its basal state can be considered in membrane-bound and cytosolic compartments (Frank *et. al.*, ١٩٩٥), the NADPH oxidase intiates the formation of reactive

oxygen intermediates (Parslow and Bainton , ٢٠٠١;Rosenberg and Gallin; ٢٠٠٣):

$$NADPH + O_2 \rightleftharpoons NADP + 2O_2^- + H^+ \quad \text{(superoxide anion)}$$

Nitrobluetetrazolium (NBT) is an electron acceptor used to detect indirectly the production of superoxide by stimulated PMNs, as outlined in the following equation:

$$NBT + O_2^- \longrightarrow \text{Formazan stippling} + O_2$$

The blue-black insoluble formazan stippling can be seen microscopically, thereby providing an assay method to screen neutrophils for the capacity to undergo oxidative metabolism (Metcalf *et al.*, ١٩٨٦).

(Park *et.al.*, ١٩٦٨) showed that the mean and range of positive NBT neutrophils were ٧.٢٪ (٥-١١٪) respectively in patients with primary TB. These findings were similar to that of AL reported in this work. Similarly, the results of NBT test of C were also in accordance to that recorded by (Fikrig and Smith, ١٩٦٨) who mentioned that the mean and range of normal subjects were ٨.٥٪ (٣-١٠٪) and (Grezegorowski *et. al.*, ١٩٩٩) who showed that the mean values was (١٢.٥ ± ٣.٠٪).

The exact role of human neutrophils to aid in defense against pulmonary infection with *M. tuberculosis* is controversial. Human monocytes/macrophages (including alveolar macrophages) fail to kill *M. tuberculosis* when infected in culture, despite incubation with cytokines or activated T-cells (Silver *et. al.*, ١٩٩٨ and Almeida *et. al.*, ١٩٩٨).

This may lead to look to other effector cells to understand innate mechanism, which protects against human pulmonary tuberculosis.

(Majeed *et. al.*, ١٩٩٨) have reported that cultured human neutrophils are able to kill an attenuated strain of *M. tuberculosis*. (Pedrosa *et. al.*, ٢٠٠٠) have suggested that murine neutrophils are protective against infection of mice with *M. tuberculosis*, although they found no evidence that the neutrophils can phagocyte the tubercle bacilli, but they indicated that human neutrophils may play an immunomodulatory role; while (Kisich *et. al.*, ٢٠٠٢) have indicated that in the examination of lung tissue from patients with pulmonary tuberculosis, neutrophils do indeed phagocytose *M. tuberculosis* in human lesions, but this ability depends directly on TNF-α , only TNF- α stimulated neutrophils can kill *M. tuberculosis*. (Jones *et. al.*, ١٩٩٠) and (Kisich *et. al.*, ٢٠٠٢) have documented that human neutrophils can kill phagocytized *M. tuberculosis* not by oxidative process, but by other mycobactericidal activities including defensins, myeloperoxidase, lactoferrin, elastase, bactericidal-permeability-increasing protein, azurocidin, cathelicidin and cathepsin-G. These mechanism may be regarded as adaptive immune response to overcome the escaping mechanisms performed by tubercle bacilli from being killing by oxidative pathway of neutrophils (Roitt *et. al.*, ٢٠٠١; Krause, ٢٠٠٠).

As mentioned above the NBT test depends on the oxidative metabolism of neutrophils. The normal positive NBT neutrophils in AL may be attributed to the normal immune functions in these patients. On the other hand; the relatively decreasing value of NBT in AN may be interpretated depending on several factors:

- Malnutrition which cause generalized immune dysfunction (Moretti, ١٩٩٢).
- Immunocompromized effect which considered one properties of anergic tuberculous patients.

- Defeciency in oxidative metabolism due to genetic defect in the *ets* family transcription factor *PU.١*, which is responsible for this activity (Anderson *et. al.*, ١٩٩٨).
- Drug effects.

NBT test; depending on the results reported in this work; may be regarded as an axillary important tool to check the non-specific cellular immunological state of tuberculous patients.

٥-٤-٢ E-rosette Formation test (T-cells count):

E-rosette is a simplified common technique used for the quantitative assay of T-lymphocytes. It is different from EAC-rosette test, which is used as a tool for the quantitative assay of B-lymphocytes. EAC-rosette test needs for the interaction of antibodies (A), complement (C), sheep erythrocytes (E) and specific ligands on the surface of B-lymphocytes, while in the case of T-lymphocytes, the interaction occurs directly between sheep erythrocytes and specific T-cell receptors ($CD_{٢}$) (Kotton, ٢٠٠٤). In addition to $CD_{٢}$, (Bernard *et. al.*, ١٩٨٨) reported that another T-cell surface molecules (٣٢kDa, ٢٠kDa and $E^{٢}$) may be involved in spontaneous rosette formation with sheep RBCs.

The result table (٧) and figure (٤) showed that there were gradual patterns for the differences in the values of E-rosette test. Generally, the values of C were higher than AN and AL, while AL values were higher than AN. At the same time, AL median values tended to be at the lower limits of the normal range, whereas AN median values were below the normal range.

(Weir, ١٩٧٣) had suggested that the majority of rosette forming cells vary during the immune response and there is a linear relationship

between the number of rosette-forming cells and the total number of lymphocytes; in other words, the rosette-forming cells is propotional to that of total numbers of the lymphocytes subsets. This means that there was a severe decreasing in T-lymphocytes count in AN and to a lesser extent in AL. This decreasing may be attributed to several factors as advocated by the following workers:

Staite *et. al.* (١٩٨٧) have reported that neutrophils may inhibit human T-lymphocytes-E-rosette formation by generation of hydrogen peroxide which may have effectiveness on the ability of T-lymphocytes to form rosette with sheep RBCs even after separation in vitro, but at the same time a proportion of T-lymphocytes appeared, for unknown reason, to be relatively resistant to this effect and they retained their ability to form E-rosette. As previously mentioned, AN patients were characterized by predominant infiltration of neutrophils leukocyte type, this may interprete, in part; the lowering rate of E-rosette forming-cells count in AN patients who seem to be have an increased number of H_2O_2–sensitive T-lymphocytes. Congenital T-lymphocytes deficiency, congenital immunodefeciency disorders, acquired immunodefeciency disorders; the most important of which is AIDS which associated directly with the most cases of TB, acute viral infection and aging may be regarded as the main causes responsible for the subnormal T-lymphocyte levels (Kotton, ٢٠٠٤). Nutritional status and genetic components of the immune system determine the generation, maturation and functioning of cellular and molecular components of the immune system. Severe protein malnutrition causes major changes in the thymus and in the peripheral lymphoid tissues. The thymus shows profound involution with marked decreases in the number of cortical lymphocytes. Peripheral lymphoid

tissues including spleen, lymph nodes, tonsils, Peyer's patches and appendix also show a significant atrophy with marked depletion of lymphocytes. Despite a normal or even increased leukocyte count in the peripheral blood, the absolute and relative number of mature T-lymphocytes is decreased, with profound decreases in the $CD_{٤}$ and $CD_{٨}$ T-cells subsets. This decreasing in T-lymphocytes number greatly contributes to the impaired cell-mediated immunity to mycobacterial infection or appearance of anergic T-lymphocytes (Dai *et. al.*, ١٩٩٨). *M. tuberculosis* as antigens have no effect on the kinetics of E-rosette reaction, but may contribute to the formation of "atypical lymphocytes" which are larger than "typical lymphocytes" and may be indented at its periphery by the surrounding cells producing a scalloped appearance, but retain their ability to form rosette with sheep erythrocytes (Simon, ٢٠٠٣).

In the view of the aforementioned discussion, one may state: The subnormal values of E-rosette forming cells count in AN may interprete the lowering immune response to *M. tuberculosis* when compared to AL who were having a relatively large number of T-lymphocytes and consequence normal immune response to mycobacterial antigens represented by the positive tuberculin skin (PPD) test which was false negative in AN. The immunological anergic state is induced in tuberulous patients when the $CD_{٤}$ T lymphocytes count is subnormal (Layne, ٢٠٠٣).

٥-٤-٣ Leukocyte migration-Inhibitory Factor (LIF) test:

Among the most popular cytokines playing a role in cell mediated immunity to tuberculosis are leukocyte migration-inhibitory factor (LIF) and other cytokines (IL-٤ and IFN- ɣ) that participate in this

functios (Bernhagen *et. al.*, ١٩٩٨). The features of a good *in vitro* method for detection of cell mediated immunity in clinics should: (١) be specific for cell-mediated immune reactions,(٢)correlates with *in vitro* manifestations of the reactions, and(٣)be quick and use a minimum of venous blood as the source for both immune reactive and indicator cells.

The inhibition of leukocytes migration from capillary tubes is one of the most popular *in vitro* methods for detection of cell-mediated immune reactions in clinics (Silobrčić *et. al.*, ١٩٧٥). The action of antigen *in vitro* upon immunocompetent cells results in an inhibition of the cells migration if the cells originate from an organism in a state of cellular hypersensitivity to the same antigen (Soborg, ١٩٦٨). Tuberculous patients table (٨) showed significant leukocyte migration inhibition indices in AL and less significant in AN patients.

These results of LIF depend generally on two main factors:
(١) The sensitivity of cells, and (٢) The antigen concentration (Soborg, ١٩٦٨). The last factor was similar in all three groups (AN, AL and C). Therefore, the variation in the results of LIF could be attributed to the first factor, sensitivity of cells.

Jüttner *et. al.* (١٩٩٨) stated that LIF plays an important role in delayed-type hypersensitivity (DTH). (Barksadle and Kim, ١٩٧٧) showed that the degree of association between LIF and tuberculin skin reaction may be more marked at the end of one year of antigenic sensitization than at the end of one month of concomitant exposure. This association may reflect the fact that tuberculin skin test is negative at first weeks of mycobacterial infection (UI health care, ٢٠٠٣).

There was a significant correlation demonstrated between the intensity of LIF and the extent of pulmonary TB and correlates well

with the tuberculin skin reaction of DTH (Trnka and Škvor, ١٩٧٩). Peritoneal macrophages taken from animals exhibiting delayed hypersensitivity are markedly inhibited from spreading *in vitro* in the presence of specific antigen (Dekaris *et. al.*, ١٩٧١).

Likewise, a sharp decrease in the mean LIF index was detected following the sensitization of rats by tuberculin (Veselić *et. al.*, ١٩٧٢). The ability of host cells to be sensitized to specific antigens determines to a large extent the differences in LIF values from patient to patient. In addition to the sensitivity of cells, other factors may affect the results of LIF including that concerning with the producer cells, LIF is produced by antigen sensitized lymphocytes, pituitary glands, cells of the brain, kidney, lung, prostate, testis and macrophages (Das, ٢٠٠٠).

Any abnormalities in the production of this factor may cause a variation in the inhibition of leukocyte migration. (Dai *et. al.*, ١٩٩٨) reported that macrophage functions including (MIF) are not impaired in protein malnutrition.

The non-significant values of LIF for C was in agreement to that reported by (Veselić *et. al.*, ١٩٧٢). These non-significant values may be attributed to the absence of long term-antigenic sensitization, which is necessary for the expression of inhibitory function.

٥-٤ Diagnostic choice:

٥-٥-١ Tuberculin skin test (PPD test):

The tuberculin skin test is used to determine whether a person has TB infection. In most persons, who have TB infection, the immune system will recognizes and interacts the tuberculin as cellular antigens because it is similar to the tubercle bacilli that caused infection, this will cause appearance of delayed-type hypersensitivity (DTH) at the site of injection (CDC, ١٩٩٥,a; Katial *et. al.*, ٢٠٠١; Menzies, ١٩٩٩).

As a reaction, tuberculin skin test is usually cited as a typical example of DTH, which mediated by T-cells. Following intradermnal injection of tuberculin, T-cell-derived TNFα and TNF β act on endothelial cell in dermal blood vessels to induce the sequential expression of the adhesion molecules E-selectin, ICAM-١ and VCAM-١. These molecules aid in the bringing of leukocytes to the site of reaction resulting in the infiltration of leukocytes starting with neutrophils after (٤ hours), but monocytes and T-cells; mainly CD٤ T-cells, are replaced after ١٢ hours. Erythema and induration develop and reach their peaks in ٢٤-٧٢ hours (Brooks *et. al.*, ٢٠٠٤; Roitt *et. al.*, ٢٠٠١).

Depending on the results of tuberculin skin test, the tuberculous patients studied in this work were classified into two main groups. The first group AN in whom the tuberculin skin test was negative, while this test was positive in the second group AL. The control subjects exhibited negative tuberculin skin test. The judgment for the positivity of this test based on the measurement of the diameter of induration area at the site of injection after ٧٢ hr, erythema (redness) around the indurated area was not measured because the presence of erythema does not indicate that a person has TB infection (CDC, ١٩٩٥,a; NC TB

control, ٢٠٠٣; Martin, ٢٠٠٣). The diameter of ≥٥ mm was considered to be positive result (CDC, ١٩٩٥,a; CDC, ١٩٩٦,a; Cohn *et. al.*, ٢٠٠٠).

Positive tuberculin skin test indicates that *M. tuberculosis* germs are in the body. Although the person is infected by TB germs, it does not mean that the person has TB disease (UI health care, ٢٠٠٣). A positive test does not indicate active disease, it is merely an exposure to the organism (Fox, ٢٠٠٤). Also, positive result indicates that an individual has been infected in the past and continues to carry viable mycobacteria in some tissues, such individuals may be at risk of developing disease from reactivation of primary infection, whereas true tuberculin negative result indicates that the person have never been infected and not subject to the tuberculosis risk (Brooks *et. al.*, ٢٠٠٤). False-positive tuberculin skin test is one drawback of this test and attributed to the following main factors:

(١) Infection with nontuberculous mycobacteria other than *M. tuberculosis*, or (٢) recent immunization with BCG (CDC, ١٩٩٥,a; CDC, ١٩٩٦,b; Sousa *et. al.*, ١٩٩٧; Pérez *et. al.*, ٢٠٠٢; Menzies, ٢٠٠٣).

Martin (٢٠٠٣) has reported that BCG vaccine has no effect on the result of tuberculin skin test in infancy. Another drawback of this test which reduce their sensitivity as an immunological parameter is a false negative result which means: Loss or reduce sensitivity or hypo-immune activity (anergy), HIV infection, measles infection and using of steroids or suppressive drugs (Layne, ٢٠٠٣; Bloomberg, ٢٠٠٤,b). Martin (٢٠٠٣) also reported that in addition to these causes, false-negative tuberculin skin test may be caused by:

* Bacterial infection (including typhoid and Brucellosis; which are commonly present at Babylon province), typhus, leprosy and pertusis.
* Metabolic disorders, especially renal failure and diabetes.

* Disorders of lymphoid organs (Sarcoidosis, lymphoma and leukaemia).

*Age extremes (elderly and newborn).

* Stress (surgery, burns and severe illness of any type).

*Recent mycobacterial infection:Tuberculin skin test gives positive reaction after ٤ weeks of infection(MCBIO ٢٠٠,٢٠٠٤) .

Other factors such as malnutrition, recent viral infection (rubella, mumps, influenza, chicken pox) and debility may cause false-negative result (NC TB control, ٢٠٠٣).

These factors reduce to a large extent the use of tuberculin skin test as a screening tool for detection of tuberculosis making that about ٢٥٪ of tuberculosios patients will have a false-negative result (CDC, ١٩٩٥,b).

In this work AN represents the group of false-negative tuberculin skin test which may be due to the factors expressed above and induction of anergy. The controls represent the group of true negative tuberculin skin test, although all of them were BCG vaccinated but their effect seem to be involuted, because this vaccine is given at once in Iraq without boosting dose. AL groups represent true positive skin test. Those patients exhibited well-defined cellular immune reaction characterized by DTH in response to intradermal injection of tuberculin.

٥-٥-٢ BCG scars:

BCG scars that have been noted in patients and controls represent a reaction of DTH to the live attenuated BCG strain (*M. bovis*). Table (١٣) showed the relatively high percentage of BCG scars in AL when compared to that noted in AN, but these percentages were high in patient groups. The absence of BCG scars may be due to: (١) The

patients might be unvaccinated, (٢) The immune system of these patients failed to responsd to this vaccine, and (٣) The scars were retrograded as time elapsed. The second factor may be one of the characteristic features of AN patients.

In general, the high prevalence of BCG scars mean that BCG; as a vaccine, does not confer a solid immunity against TB. Thus TB may be reported in scar bearing vaccine as the present work indicated. (Sousa *et. al.*, ١٩٩٧) found the BCG scars in ٧٦٪ of tuberculous patients.

The methods and routs of vaccine administration, viability, shelf life and potency may affect the efficacy of this vaccine. Recently, intranasal mucosal administration of BCG vaccine improved protection against TB greater than subcutaneous vaccination (Chen *et. al.*, ٢٠٠٤). The environment and host factors that characterize population vaccinated with BCG may also affect this efficacy (CDC, ١٩٩٦,$_b$).

٥-٥-٣ Demonstration of AFB:

Acid fastness is defined as the ability of the bacterial cell to resist decolorization by weak mineral acids after staining with one of the basic dyes. This property is widely used for the microscopic detection of mycobacteria in clinical specimens. Other related bacteria especially *Nocardia* may be partially acid fast. The property of acid fastness depends mainly; as a chemical reaction, on the mycolic acids or lepronic acid which is the basic subunit of lipids in the cell wall of Mycobacteria. The mycolic acids arrange in certain configurations that cause a trapping of the dye (Grange, ١٩٨٨; Beaman, ٢٠٠٣). Positive AFB smears mean active infections TB case, but a negative smear does not rule out the possibility of TB because, or patients who have negative smears may have TB (CDC, ١٩٩٥,$_a$). Although, acid-fast

smear is not specific for *M. tuberculosis*, the positive predictive value of AFB for *M. tuberculosis* in respiratory specimens is high (more than ٩٠٪) and the prevalence of nontuberculous Mycobacteria is low. (Conde *et. al.*, ١٩٩٩; Pfyffer *et. al.*, ٢٠٠٣).

The results table (١٣) showed the low rate of AFB in both groups of tuberculous patients. This was due to the effect of drugs (Pfyffer *et. al.*, ٢٠٠٣) given for tuberculous patients. This finding was in accordance with (CDC, ١٩٩٥,b) which recorded that only ٤٠٪ of tuberculous patients have positive AFB smears.

٥-٥ Anergy, a practical or operational concept:

The maintenance of homeostasis and the induction of an immune response whether tolerogenic or immunogenic, relies on an efficient immune system that distinguishes tissue-specific self-antigens from foreign antigens. T-cell-tolerance to self-antigen appears to be an important physiological mechanism to prevent autoimmune diseases, whereas allowing protective immune response to non-self antigen (or pathogens) is achieved by: (١) clonal deletion, (٢)T-cell anergy, and (٣)induction of regulatory cells (Avice *et. al.*, ٢٠٠١).

T-cell anergy is characterized by the inability of the T-cell to produce IL-٢ and proliferate (Erman *et. al.*, ٢٠٠١). Many researchers to express the anergy as an immunological state have studied several mechanisms (٢.٩). The key factors for the induction of T-cell anergy are depend directly on the interaction between APCs and T-cells. Collectively, $CD_{٢٨}$, and CTLA-٤ on the surface of T cell play a central role in the induction of T-cell anergy. Likewise; T-cell anergy may be induced as a result of deficiency in AP-١ transcriptional factor, which is necessary for the production of IL-٢ (Sundstedt and Dohlsten,

١٩٩٨). Eosinophils growth and proliferation depends on the activity of IL-٤, IL-٥ and IL-١٣ which are screted by activated $T_{H٢}$ cells, therefore anerginized (inactivated) $T_{H٢}$ cells may diminish the growth and proliferation of eosinophils (Oppenheim and Russcetti, ٢٠٠١).

Anergy as a phenomenon may be of beneficial effects in case of self-antigen unresponsiveness and transplantation, or of harmful effect in case of non-self antigen unresponsiveness which may increase the severity of disease.

In this work, AN patients were suffering from hyporesponsive state to mycobacterial antigens resulting in the acute prognosis of tuberculosis when compared to AL patients. In addition to increasing the acuteness of the disease, anergy make several problems in the diagnosis of tuberculosis which depends mainly in Iraq; due to the unavailability of the modern diagnostic techniques, on tuberculin skin test which gives false-negative result in AN patients. Therefore, this group of anerginized patients needs to more sensitive tests to evaluate the pathological state of tuberculosis. The non-specific parameters used in this work included NBT and systemic and local differential leukocyte count. These non-specific parameters appear to be as non-specific probes for the diagnosis of tuberculosis. Although, NBT test gave a gradual variation among the studied groups, but the median of both AN and AL stilled in the normal range. On the other hand; specific parameters have included AFB, tuberculin, BCG, LIF and E-rosette tests. As discussed previously (٥.٥.٣) AFB negative smear does not mean the absence of tuberculosis in both patient groups. Likewise, tuberculin skin test, False-negative result is the main characteristic feature of AN patients, False-positive result may also make a great degree of diagnostic interference-BCG scars appeared on both patients

groups. Therefore, these three specific parameters could not be used as sufficient tools in the diagnosis of tuberculosis and to determine whether the host immunological state is of anergic or allergic type. LIF and E-rosette showed a high level of clear-cut differences between AN and AL, thus these two specific parameters may be used as cellular immune parameters to discriminate between AN and AL.

CONCLUSIONS

١- Anergy may be in association with local and/or systemic eosinophilia and basophilia.

٢- Lymphocytosis or monocytosis were rather common among anergy.

٣- Anergy may fall in one of the following entities:

i) Clinical.

ii) Cellular, and

iii) Clinical-cellular anergy.

٤- Anergy may represent a reduction in cellular immune function in one or more than one type of immunocompetent cells, as macrophages and various T-cells subsets.

٥- Non or less significant LIF values were associated with anergy.

٦- Subnormal E-rosette and NBT values were associated with anergy.

٧- LIF and E-rosette can be a “Test Battery” of choice for evaluation of cellular immune function in tuberculous anergy.

RECOMMENDATIONS

Performing further investigations for the anergic tuberculous patients such as role of:

i: $CD_{۲}$ T-cell count.

ii: Checking HLA-DR types.

iii: Checking mucosal T-cell function.

iv: Alternative mycobacterial antigens for skin to overcome the cross reactivity observed in tuberculin (PPD) skin test.

v: Cytokines such as IL-۲ and IFN-□ as a mediators able to reverse the anergy.

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