# IMMUNOHISTOCHEMICAL STUDY OF TUBERCULOUS LYMPHADENITIS

**A**

**Research work**

**Submitted to the Nepal Health Research Council**

**By**

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# COMPARATIVE EVALUATION OF DIFFERENT STAINING TECHNIQUES FOR THE DIAGNOSIS OF TUBERCULOUS LYMPHADENITIS

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# RECOMMENDATION

This is to certify that **Ms Smritee Pokharel** has completed this dissertation work entitled **"COMPARATIVE EVALUATION OF DIFFERENT STAINING TECHNIQUES FOR THE DIAGNOSIS OF TUBERCULOUS LYMPHADENITIS"** as a partial fulfillment of M. Sc. Degree in Microbiology. To the best of our knowledge, this is an original work of her and has not been submitted for any other degree.


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# ABSTRACT

This study was conducted at Patan hospital, during September 2002 to March 2003 in joint collaboration with Central Department of Microbiology, Tribhuvan University with the objective to evaluate different staining techniques for the diagnosis of tuberculous lymphadenitis on clinically suspected cases. Altogether, 40 biopsies collected at Department of Pathology, Patan Hospital were further analyzed. 40 biopsy specimen were stained with Haematoxylin - Eosin stain and Acid fast stain where as 20 biopsies were stained with CD3/ CD20/ S-100 stains respectively and the biopsy cell/tissue features were analyzed.

Among 40 cases of suspected tuberculous lymphadenitis cases, 100% cases showed tuberculosis positive in Haematoxylin-Eosin stain. Where as Acid Fast Bacilli could be detected in only 10% of the cases. Greater prevalence of tuberculous lymphadenitis was observed between 20-30 years of age with higher percentage of female's involvement. Frequency of Cervical and axillary nodes involvement were higher than other. In 57.5% of cases, right cervical nodes and in 20% of the cases, axillary nodes were found involved. Multiple nodes involvement was observed in 80% of the cases and bilateral nodes in only 20% of the cases. CD3 cells were present in higher numbers than CD20 cells. CD3 cells were confined to paracortical areas where as higher CD 20 cells were found in follicular area. But due to migratory nature of CD20 cells, they were also found in other parts of lymph nodes.

Immunohistochemical staining techniques, though specific, H-E staining and AFB staining in combination still remains a method of choice for the diagnosis of tuberculous lymphadenitis, in developing country like Nepal, because of cost benefit and availability of immunohisto-chemical staining reagents.

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# ABBREVIATION

| | |
|---|---|
| AFB | Acid fast bacilli |
| AIDS | Acquired immuno deffiency syndrome |
| BCG | Bacillus Calmette Geurine |
| CD | Cluster designation |
| DNA | Deoxyribonucleic acid |
| ESR | Erythocyte sedimentation rate |
| FNA | Fine needle aspiration |
| FNAC | Fine needle aspiration cytology |
| HIV | Human immunodeficiency virus |
| HMG | His Majesty's Government |
| INH | Isoniazid Hydrochloride |
| LJ | Lowenstein-Jensen |
| MHC | Major Histocompatibility Complex |
| No. | Number |
| NTC | National Tuberculosis Control |
| NTP | National Tuberculosis Programme |
| PCR | Polymerase chain reaction |
| PMNs | Polymorphoneuclear cells |
| PPD | Purified protein derivative |
| ppm | Parts per million |
| RBC | Red blood cell |
| SAARC | South Asian Association for Regional Co-operation. |
| TB | Tuberculosis |
| TU | Tuberculin unit |
| WHO | World Health Organisation |
| Z-N | Ziehl-Neelson |
| IRCs | Interdigitating reticulum cells |
| DRC | Dendritic reticulum cells |

# 1. INTRODUCTION

Tuberculosis is chronic bacterial infection caused by *Mycobacterium tuberculosis* and characterized by the formation of granuloma in infected tissue as a result of cell mediated hypersensitivity (Thomas, 1994).

Tuberculosis is among the top ten causes of global mortality. It has been estimated that approximately one-third of the world's population is infected with the tuberculosis bacillus, and that each year 8 million people develop the disease and 1.8 million die of the disease. Approximately 80% of tuberculosis cases are found in 23 countries; highest incidence rates are found in Africa and South-East Asia (Borgdorff, 2002).

Tuberculosis as a killer disease has probably been recognized since the stone age. Traces of tuberculous lesion have been found in the lungs of 3000 year old Egyptian mummies. Today the co- epidemic of tuberculosis and HIV is a major problem in the world. HIV increases the risk of getting tuberculosis 30-50 times (STC, 2000).

Nepal presenting an exemplary scenario has about 45% of its total population infected with Tuberculosis, out of which 60% are in the economically productive age group of 15-49 years. Even after the implementation of a highly cost effective & extremely successful treatment strategy of DOTS (Directly Observed Treatment Short Course) as a part of National Tuberculosis Program (NTP) in April 1996, around 44,000 Nepalese develop active tuberculosis every year, of whom 20,000 have the infectious pulmonary form claiming 6,000 to 7,000 lives annually ( www.stoptb.org/, 2003).

Tuberculosis can occur in any part of the body and most common site of infection is lung causing pulmonary tuberculosis. In extra pulmonary cases peripheral lymph nodes are the commonest sites, among them 70% to 90% occur in cervical lymph nodes (Basnet, 1998). Lymphadenitis is acute and chronic inflammatory process of lymph node that occurs in response to a variety of pathogenic agents. They may be specific or nonspecific, featuring necrosis, abscess, granulomas and fibrosis in various combinations (Ioachim, 1994).

Tuberculous lymphadenitis is a rare condition in Western countries. However, in developing country like Nepal, where tuberculosis is still rampant, tuberculous lymphadenitis continues to be one of the most common types of lymphadenitis (frequency 30.0-50.6%) encountered in clinical practice (Bhanot, 1999).

Cases of Tuberculous lymphadenitis are detected on the basis of clinical findings, radiological findings and laboratory results. Clinical parameters include low grade evening fever, tenderness, and enlargement of the lymph nodes. Radiological findings may not be specific in the diagnosis of lymph node tuberculosis. Laboratory diagnosis includes microscopy of lymph node biopsy or the lymph node fluid obtained from Fine Needle Aspiration utilizing the Haematoxylin-Eosin, Ziehl-Neelson or fluorescent stain. Though culture can be done on grinded tissue specimen or the aspirates, due to longer generation time of *Mycobacterium tuberculosis*, it is not in common use. Rapid detection of *Mycobacterium tuberculosis* via BACTEC can also be done. But with the immergence of newer diagnostic tools like Polymerase chain reaction (PCR) and Enzyme linked immunosorban assay (ELISA), early diagnosis has been possible. Also that, during tuberculosis infection, changes in the activity of cells involved in immune response (T and B cells) occurs, which could also be measured and changes could be monitored as a part of diagnosis or effectiveness of treatment.

Management of tuberculous lymphadenitis involves appropriate use of antituberculous chemotherapy with the judicious use of surgical excision in a minority of patients (Powell, 1999).

Diagnosis of tuberculous lymphadenitis on the basis of clinical finding in combination with Fine Needle Aspiration Cytology (FNAC) of the lymph node aspirate or hematoxylin-eosine staining of the lymph node biopsy is common in practice. Additional staining of the specimen by Ziehl-Neelson (Z-N) stain may provide a step toward better diagnosis of the cases. Cause of Z-N stain not common for the diagnosis may be due to the fact that only lower number of bacilli are present in the lymph node biopsy and its detection is very time consuming as well as tedious. Use of alternate method for the diagnosis of tuberculous lymphadenitis may be essential, thus

immunohistochemical staining of the lymph node biopsies in combination with Z-N stain may yield a better diagnosis specially in case of lymph node tuberculosis.

Study of the immunological changes like effect on T cells/ B cells/ on the lymph nodes during the infection period could be useful in early diagnosis of tuberculous lymphadenitis. Keeping all these factors in mind, this work has been planned to evaluate the efficiency of immunohistochemical staining in the diagnosis of tuberculous lymphadenitis.

# 2. OBJECTIVES:

## 2.1. General Objective:

Use and evaluation of different staining techniques in the diagnosis of tuberculous lymphadenitis.

## 2.2. Specific Objective:

1. To determine the sensitivity of immunohistochemical staining targeting toward staining CD3, CD20 and S-100; in the diagnosis of tuberculous lymphadenitis.

2. To determine the sensitivity of histochemical staining (Hematoxyline–Eosin) in the diagnosis of tuberculous lymphadenitis.

3. To determine the sensitivity of AFB staining in the diagnosis of tuberculous lymphadenitis.

4. Comparative evaluation of immunohistochemical/ AFB and H-E staining in the diagnosis of tuberculous lymphadenitis.

# 3. LITERATURE REVIEW:

## 3.1. Definition:

Tuberculosis is a chronic bacterial infection caused by *Mycobacterium Tuberculosis* and characterized by the formation of granuloma in infected tissue as a result of cell-mediated respense. In majority of the cases it affects lungs causing pulmonary tuberculosis. But may also have extra-pulmonary extension affecting the lymph nodes, intestine, meninges, bones and joints, skin and other parts of the body with varying clinical manifestations such as, evening rise of fever, decreased appetite and weight loss, haemoptysis and progressive weakening of the body.

## 3.2. Historical Background:

Tuberculosis is a disease of great antiquity having been identified in mummies from the fourth million B.C. When and where the battle with the disease began remains a matter of conjecture but there is evidence that *Mycobacterium tuberculosis* must have preceded the recorded history (Basnet, 1998).

The clinical features of both pulmonary and spinal tuberculosis were well described by Hippocrates in about 400 B.C. Accounts of the diseases appeared in the Vedas and other ancient Hindu texts, in which it was sometimes termed Rajyachhyama (Meaning the king of Maladies in Sanskrit), the king of diseases and it afflicted Neolithic man and pre Columbian Amerindians (Grange, 1990).It is the Hippocratic collection that describes the first authentic account of clinical tuberculosis. Scattered throughout the volumes are numerous references to Pthisis the term which Greek physicians introduced to describe the disease accompanied by progressive weight loss. (Basnet, 1998)

The transmissible nature of tuberculosis was clearly established by Jean – Antoine Villemin, a French military Doctor. In 1868, Villemin published the result of a series of studies in which he convincingly demonstrated that tuberculosis could be produced in rabbit by inoculating them with tuberculous material from man or cattle. The disease could be passed from animal

to animal and differences in virulence were observed between human and bovine material. In addition Villemin established that Scrofula (tuberculous cervical lymphadenitis) and pulmonary tuberculosis were different manifestations of the same disease (Grange, 1990).

Villemin's prediction that the causative agent of tuberculosis could be isolated was realized in1882 when Robert Koch succeeded in culturing the bacilli on inspissated serum. In addition to culturing the causative organism, Koch succeeded in staining it by treatment with alkaline solution of methylene blue for 24 hours. The technique was subsequently improved by Ehrlich by using a hot solution of the arylmethane dye fuschin and it is this technique, slightly modified by Ziehl and Neelsen whose name it bears, that is still used today (Grange, 1990).

Though the disease has been identified earlier, the modern era of tuberculosis treatment began only in 1946 with the advent of streptomycin and in 1952 A.D. with development of Isoniazid Hydrochloride (INH). Since than the modalities of treatment regimens were constantly revised and updated. At present, giving short course chemotherapy of six months duration treats tuberculosis.

## 3.3. Epidemiology:

### 3.3.1Global situation:

Murray *et al* (1990) estimated a case rate of 229 per 1,00,000-population death in Sub Saharan Africa due to tuberculosis. Where as, Kochi (1991) estimated a case rate of 272 per 1,00,000 population, in African region. Similarly Murray *et al* (1990) estimated that there were 2.5 million deaths from all forms of tuberculosis in developing countries. Kochi (1991) estimated that worldwide, tuberculosis caused 2.9 million deaths in 1990. Both estimate showed tuberculosis to be the largest cause of death from a single pathogen in world, out of which, 1.7 to 1.8 million deaths occurring in Asia only (Bhatta CP, 1996).

The largest annual numbers of cases are from South- East Asia, accounting for almost half of the total cases in the world. However, the incidence rate is estimated to be highest in Africa

and the lowest in industrialized countries. It was reported that nearly 95% of tuberculosis death were in developing countries (Rieder, 1999).

In USA, of all newly detected tuberculosis cases, 5% are those of tuberculous cervical lymphadenitis. Ninety- percent tuberculous cervical lymphadenitis is unilateral and 90 % involve one node group. Any of the cervical nodes may be involved, but the most common are the nodes of the deep jugular chain, followed by those of the sub-mandibular region and then of the posterior triangle. Lymph nodes other than those in the cervical region are less commonly involved in tuberculosis and account for 35% of tuberculous adenitis (Baskota *et al.* 1995).

**3.3.2. Situation in Nepal:**

Tuberculosis is an immense problem in Nepal, causing great suffering and death. Recent estimates suggest that about 45% of the total populations are infected with tubercle bacilli and each year about 50,000 people develop tuberculosis, over 20,000 of who have infectious sputum smear positive disease. According to the recent estimates, about 80,000 to 90,000 people in Nepal have active tuberculosis and annual death is about 8,000 to 11,000 (NTC/STC, 1999).

In Nepal five years ago, it was estimated that about 16,000 people were dying from tuberculosis every year. Current estimates have shown a profound decline in the number of deaths to about 8,000 per year. This decline in death rate is due to improvements in program performances to control tuberculosis. (NTC/STC,1999).

In Nepal, majority cases are in rural areas where more than 90% of population resides. The annual rate of infection is estimated at about 3%. In hilly area it is about 1.5%, in terai area it is about 2.5%, in urban area it is about 4% and in mountain area is less than 1 % (Amatya, 1992). Although numbers of pulmonary cases are more than extrapulmonary tuberculosis, 42.72 % in Dhunkuta, 38.89 % in Terhathum and 25.45 % in Taplejung were the extra pulmonary infection. In one study it was found that out of 349 cases of tuberculosis diagnosed histopathologically, lymph node tuberculosis was found to be the commonest (66.3 %),

(Shrestha, 1989). Also in a study conducted in Kanti Hospital has 17% cases of glandular tuberculosis specially affecting cervical, axillary, inguinal and abdominal glands.

In a study conducted on Eastern Region of Nepal, it was found that tuberculous lymphadenitis is the commonest cause of cervical lymphadenopathy though metastatic carcinoma was also found in 18% of the cases (Thakur, Prakash *et al.* 1998). So, tuberculous lymphadenitis continues to be one of the most common types of lymphadenitis (frequency 30.0-50.6%) encountered in clinical practice (Bhanot, Raut *et al.* 1999).

## 3.4. Anatomy and Physiology of lymph node:

In the lymphatic pathway, lymph nodes are peripheral lymphoid organs connected to the circulation by afferent and efferent lymphatic. Ovoid or round, bean shaped nodules composed of dense accumulations of lymphoid tissues; vary in size from 2mm to 20 mm and average 15mm in longitudinal diameter. The major function of lymph nodes is lymphopoiesis, filtration of lymph, and processing of antigens (Ioachim, 1994).

The lymph node cells are arranged in a delicate meshwork of the reticulum surrounded by the capsule. In the periphery of node, there is a closely packed layer called the cortex. The cortical lymphocytes form spherical lymphatic nodules. Uniform and tightly packed nodules are called primary follicles. After encounter with antigen, germinal centers can be seen in the nodules, which are than identified as secondary follicles. The germinal center of each secondary follicle is surrounded by a zone of small lymphocytes called the mantle. The cortex area in the lymph node is divided into the nodular cortex, containing the follicles and the diffuse cortex. The follicles contain B-lymphocytes, while the diffuse cortex is rich in T-cells. The cortex also contains accessory cells which express class II MHC determinants on their surface and specialize in antigen presenting function. Most plasma cells and phagocytic cells are located in the medulla. Upon antigenic stimulation, the regional lymph node shows proliferation of T cells in the diffuse cortex and of B-cells in the follicles. The secondary follicles with active germinal centers contain dendritic antigen presenting cells, macrophages and CD4+ T lymphocytes.

According to the concepts of Lukes and Collins, the follicular center, a major component of the B -cell system, contains in addition to dendritic reticular cells and tangible body-macrophages, four types of lymphoid cells (centrocytes) and small and large cleaved lymphoid cells (centroblast).Their relative proportions vary in relation to the degree of immunologic activity of the follicle. The small B-lymphocytes in the peripheral mantle are stimulated by antigens to transform and undergo blastic transformation. The first stage of transformation is represented by the cleaved lymphoid cells, which are the non-dividing forms. Gradually the small-cleaved cells acquire a narrow rim of pyroninophelic cytoplasm as they reach the large cleaved cell stage. Further, the nuclear cleavage disappears, as the nuclei become round or oval, about four times the size of the small resting lymphocytes. The cytoplasm is abundant containing multiple cell organelles, and the nucleoli are prominent. Mitoses of these cells result in small non-cleaved cells that further mature into plasma cells. The non-cleaved lymphoid cells are the dividing forms of the follicular center cells. Normal lymph node contains 20% to 35% immunoglobulin bearing cells in cell suspensions, an amount that may increase to 80% in some reactive follicular hyperplasias and to 100% in monoclonal B cell lymphomas. (Valk and Meijer, 1992).

Since we are in constant contact with antigens, a lymph node will always show some degree of stimulation. Depending on the kind of antigen challenge, one or more of the compartments is stimulated, which results in an increase in the volume of the compartment or a shift in its cellular composition. In the latter instance, an increase in blast cells is often seen. Since blasts represent the proliferative phase of a reaction, their increase leads to expansion of the entire compartment. This adaptability to the challenging antigen explains the variability in normal lymph node histology (Valk and Meijer, 1992).

Due to the important role in dealing with antigens that is, the immune response, that makes the lymph node such an intriguing organ. For instance, reaction to certain macro molecules with repetitive features in their overall structure, such as lipopolysaccharide, are mediated by B lymphocytes independent of T lymphocytes, where as reaction to most soluble antigen are T-cell dependent; in addition, some reaction to particulate antigen require primarily T lymphocytes. This is reflected in lymph node histology. Immunological reaction takes place in

specific compartments of lymph node that is the follicles, the medullary cords, the paracortex and the sinuses.

## 3.5. Disease:

### 3.5.1. Tuberculous Lymphadenitis:

Lymph node involvement constitutes the most common presentation of extra pulmonary tuberculosis (Bhanot, Raut *et al.* 1999). Tuberculosis of peripheral lymph nodes is the commonest form of extra pulmonary tuberculosis and frequently involves the lymph nodes draining head and neck. It may be a part of extra pulmonary tuberculosis or it may be the exclusive involvement confined to a particular group of lymph nodes (Basnet, 1998). Cervical lymph nodes are the most frequently involved extra thoracic lymph nodes throughout the world. The disease in this location was also known as "Scrofula" or the Kings evil.

Lymph node tuberculosis is the primary infection of tuberculosis. In most of the cases the primary complex is asymptomatic and undergoes spontaneous healing by the resolution, fibrosis or calcification resulting in hypersensitivity to tuberculoprotein i.e, tuberculin allergy and some degree of acquired resistance (immunity). Occasionally primary infection may progress by direct extension or may dissiminate leading to miliary tuberculosis, meningitis, bone and joint tuberculosis, renal and endometrial tuberculosis. Lung infection is the secondary infection and in secondary infection, lymph nodes are slightly enlarged and shows no caseation necrosis. (Chakrabourty, 2000).

### 3.5.2. Aetiology:

The causative organism is the *Mycobacterium tuberculosis*. Robert Koch explained the causative role in tuberculosis by satisfying Kochs postulates. The microorganism belongs to the genus Mycobacteriaceae of the order Actinomycetales. There are more than 50 Mycobacterium species including many saprophyte *Mycobacterium tuberculosis*. These are usually slightly curved rod shaped organisms with parallel sides and rounded ends, usually 1-4 um $\pm$ 0.3-0.6 μm in size, which frequently forms small clumps. Cells of *Mycobacterium*

*tuberculosis* are often arranged in serpentine cords. They are called mycobacteria as they resemble fungal culture (Grange, 1990).

### 3.5.3. Pathogenesis:

Lymph node tuberculosis occurs either by primary infection in the tonsils or as a reactivation of previously contained foci in lungs or by extension of contagious focus. In primary infection when bacilli reach the tonsil, pharyngeal lymphoid tissue or the alveoli, a number of specific inflammation occurs followed by phagocytosis of replication. Lymphatic involvement is believed to be an integral part of the tubercular infection with generalized lymphatic and hematogenous spread rather than a localized process.

When there is spread of infection in the cervical lymph node then there will be periadenitis and multiple lymph nodes are matted together. After sometime caseation necrosis occurs, leading to cold abscess. Caseation necrosis may be due to the phosphatids present in the cell wall of the bacilli. Later on ulceration in skin leading to discharging sinus and the pus may cause dermatitis, which is called scrofuloderma. So tuberculous lymphadenitis develops in four stages: -

1. Stage of lymphadenitis.
2. Stage of caseation (cold abscess, collar stud abscess).
3. Stage of periadenitis (matted nodes)
4. Stage of ulceration (sinus formation).

So patient may present at any stage of the disease.

### 3.5.3. a. Mode of infection:

The infection is usually transmitted from person to person by the inhalation of droplet nuclei measuring 1-5 micron and drinking of infected milk, which is not so common nowadays. The bacilli most commonly reach in the lymph node by lymphatic channel. Cervical lymphadenopathy may be a primary complex usually involving the tonsillar nodes or it could be due to hematogenous spread. Usually tuberculous lymphadenitis is associated with silent initial infection of a tonsil; and then it spreads to cervical lymph nodes.

### 3.5.3. b. Risk of infection:

Risk of infection is determined by closeness of contact, source of infection and the immunostatus of the host. Risk especially increases in crowed and poor ventilated area (Basnet 1998). A high incidence of this extrapulmonary disease has become synonymous with the human immunodeficiency virus infection (Davis, 1994) in Zimbabwe and Ethiopia, tuberculosis is found at presentation in approximately one third of patient infected by HIV-1. Positive serology is also found in 17-55% of tuberculosis patients in several central and east African countries. So one of the most common risk factor for tuberculosis is the HIV infection. (Basnet, 1998). Also the vulnerable groups or the individuals more susceptible to the disease include persons under conditions like, poorly controlled diabetes mellitus, chronic lung disease (bronchitis) and silicosis, cancer, advance kidney disease, malnutrition, alcoholism, disease for which steroid therapy is prescribed; heavy smokers, elderly, low income groups, intravenous drug users, living and attending drug treatment centers, hospital, nursing homes etc. (WHO, 2000).

### 3.5.3. c. Spread of disease:

1. Direct extension,
2. Lymphatics and
3. Blood stream

The spread through lymphatic is the most important one. Tuberculosis is a disease of the lymphoid tissue and the pharynx or intestine and in tracheobronchial and mesenteric lymph nodes usually the organisms pass through the lymphatics to the regional lymph nodes. Where they are filtered and a condition of lymphnoditis occurs as a result of primary infection in case of pulmonary infection, tracheo bronchial lymph nodes are involved. In intestinal infection acquired by ingestion of the organisms, mesenteric lymph nodes are affected. By passing through the lymph nodes barrier of the mesenteric nodes, the organisms may pass to the thoracic duct, enter into the blood stream and reach the lungs. Here it may be arrested producing a focus of infection. Where as, lymphatic spread occurs regularly as a biological law during the primary infection (primary complex) the process being absent or inconspicuous in the re-infection type (Bhatta, 1996).

Ingestion of contaminated cow's milk, once considered as a common cause, has now become an insignificant mode of spread. (Basnet, 1998)

## 3.6. Virulence factors and their interaction with host cells:

Mycobacterium tuberculosis is a facultative, intracellular parasite that infects and multiplies, primarily within professional phagocytes. Mycobacterial virulence factors can have both direct and indirect effects on host cells and can ultimately lead to tissue destruction and disease. Direct cell-cell interactions, including attachment, invasion, and intracellular multiplication, and indirect interactions, through secreted bacterial factors such as hemolysin and cytotoxin, can cause lysis of the host cells. In addition, lipoarabinomannans (LAMs), heat shock proteins, and mycobacterial products can stimulate host cells to produce inflammatory products or cytokines that can amplify tissue damage in the host (Quinn, Newman, et al. 1996).

**Table: 3.1. Known and suspected virulence factors of *M tuberculosis*.**

| Putative factors | Proposed activity | References |
|---|---|---|
| Invasion protein | Invasion of nonprofessional phagocytes and intracellular survival | Arruda *et al*. 1993 |
| Fibronectin binding proteins | Adhesion and invasion | Schorey *et al*. 1995 |
| Complement and mannose recepter binding | Entry into professional phagocytes | Schlesinger *et al*. 1990 |
| Alternate phagosomal pathway(phagosomal trafficking) | Survival in the phagosome | Clemons and Horowitz, 1995 |
| Prevention of phagolysosome fusion | Survival in the phagosome | Armstrong and Hart, 1971 |
| Escape into the cytoplasm and budding into novel phagosome | Survival in the cytoplasm | Mc Donough *et al.* 1993 |
| Hemolysin/ phospholipase | Lysis of phagosome; leakage of the phagosome | Leao *et al.* 1995 |
| Cytotoxicity | Tissue distruction and enhanced TNF effects | Mc Donough and Kress 1995 |
| Lipoarabinomannons | Reduced TNF induction and macrophage effector function | Roach *et al.*1993; Chatterjee *et al.*1992 |
| Heat shock proteins | Induction of inflammatory cytokines | Retzaff *et al*. 1994 |
| Cord factor | Tissue distruction and necrosis | Behling *et al.* 1993 |
| Sulfolipid | Induction of inflamatory mediators | Zhang *et al.* 1988 |
| Superoxide dismutage catalase | Inhibits reactive oxygen and nitrogen radicals within the phagosome | O'Brein *et al.* 1994 |
| Exochelins mycobactin | Acquisition of ferric iron | Gobin *et al.* 1995 |

TNF: Tumor necrosis factor
Source: Quinn, Newman and King (1996); (with modification).

## 3.7. Immunology:

### 3.7.1. Entry of *M tuberculosis* in mononuclear phagocytes

The earliest interaction between *M tuberculosis* and the mononuclear phagocyte is binding of the bacterium to the cell surface and subsequent internalization. Specific receptor-ligand interactions mediate this internalization but the outcome of one interaction may be different than the outcome of another. Apart form receptor-ligand interactions, the fate of pathogen is dependent upon the microbe's unique surface molecules, which either by themselves or by binding to specific host molecules can influence the host cell response during entry. Electron microscopy studies have shown that entry of *Mycobacterium tuberculosis* into human mononuclear phagocytes resembles conventional receptor mediated phagocytosis in which phagocyte pseudopodes move circumferentially around the bacterium and fuse at their distal tip, leaving the bacterium in a membrane bound vacuole or phagosome (Schlesinger, Bellinger *et al*. 1990).

### 3.7.2. Consequence of phagocytosis of *M tuberculosis*:

Entry of *Mycobacterium tuberculosis* via the CR pathway may provide the bacterium safe passage into mononuclear phagocytes by allowing it to avoid the toxic consequences of the oxidative burst. Mycobacteria including *Mycobacterium tuberculosis* also have surface glycolipids that scavenge toxic oxygen radicals, providing another mechanism for enhancing the survival of these bacteria during phagocytosis.

### 3.7.3. Mechanism of Macrophage Activity against *Mycobacterium tuberculosis*:

The antimicrobial mechanisms of macrophages often are classified into two groups on the basis of Oxygen requirement: they are said to be oxygen dependent and oxygen independent (Lowrie and Andrew, 1988). But it is also found that the effective mechanisms may change between types of macrophages.

**Oxygen – Dependent Mechanisms:**

Phagocytosis by polymorphonuclear and mononuclear phagocytes is accompanied by cyanide insensitive increase in the consumption of oxygen. This is the respiratory burst (Stahelin, Suter *et al*. 1956). During the respiratory burst there is a one-electron reduction of molecular oxygen to further interaction between these species can lead to the formation of other reactive forms of oxygen; hydroxy radical and singlet oxygen (Halliwell and Gutterridge, 1984). The electrons are derived from NADPH and formation of super oxide is catalyzed by a multicomponent redox system, the NADPH oxidase. Information in the structure of the oxidase and its role in antimicrobial activity of phagocytes has been reviewed recently (Dinauer and Orkin 1992). It has been suggested that, in addition to the directly toxic nature of its products, the NADPH oxidase has a microbicidal activity by its involvement in modulation of the $P^H$ within the phagocytic vacuole (Henderson,Chappell *et al*. 1988).

In humans, the evidence is all circumstantial. Individuals whose macrophages are defective in a respiratory burst can develop a disseminated infection when vaccinated with *Mycobacterium bovis* BCG (Mackay *et al*.1980). Furthermore, it has been suggested that peroxide susceptible strains of *Mycobacterium tuberculosis* may be less virulent in humans (Tripathy *et al*. 1969).

**Oxygen-independent Mechanisms:**

Substances making up the oxygen-independent antimicrobial mechanisms is far more extensive than those forming the oxygen-dependent systems. Various enzymes, peptides, organic acids and lipids present in macrophages and other cells can kill *Mycobacterium tuberculosis*. Macrophages possess cytoplasmic granules (lysosomes) containing a large variety of hydrolytic enzymes. Although the majority of these enzymes have a role in digestion rather than killing of microorganisms, lysosomes do contain material capable of killing microorganisms (Andrew, *et al.* 1985).

Antimicrobial proteins and peptides exhibit an antimicrobial activity that is not dependent on enzymatic activity. They have been described as part of the antimicrobial system of neutrophils, macrophages and intestinal epithelium. Lysosome of human neutrophils also contains such proteins (Spitznagel, 1990).

### 3.7.4. The role of Macrophages:

When *Mycobacterium tuberculosis* is inhaled into the lung, they are engulfed by alveolar macrophages, which perform three important functions. First, they produce proteolytic enzymes and other metabolites, which exhibit mycobactericidal effects. Second, macrophages process and present mycobacterial antigens to T lymphocytes, including CD4 and CD8 T lymphocytes, which are central to acquired resistance to *Mycobacterium tuberculosis.* Third, macrophages produce a characteristic pattern of soluble mediators (cytokines) in response to *Mycobacterium tuberculosis* that have the potential to exert potent immunoregulatory effects and to mediate many of the clinical manifestations of tuberculosis.

Intrlukin-1 consists of two structurally related polypeptides IL-1α and IL-1β, both of which have similar spectrum of biologic activities. IL-1 is produced upon stimulation of human monocytes with *Mycobacterium tuberculosis,* lipoarabinomannan present in cell wall of the organism and mycobacterial proteins with molecular masses 20 and 46 kDa. Il-1 is an endogenenous pyrogen and may contribute to fever, that is characteristic of tuberculosis. In addition, IL-1 may enhance the inflammatory response by inducing macrophages to produce IL-6 and tumor necrosis factor (TNF) and by stimulating T cell proliferation.

IL-2 causes T cell division and is likely to expand population of antigen-reactive T cells, increasing the local concentration of macrophage activating factors secreted by T cells. In contrast to the effects of Th1 cytokines, IL-4 deactivates macrophages and blocks T cell proliferation by down regulation of IL-2 gene. IL-4 therefore has the capacity to inhibit the immune response to *M tuberculosis.*

Tumor Necrosis Factor (TNF) is also produced in response to mycobacterium and its components like lipoarabinomanan and proteins. TNF can exhibit both protective and pathologic effects in *Mycobacterium tuberculosis* infection. Local release of TNF at the site of disease contributes to granuloma formation, control of infection and mycobacterial elimination. Excessive local production of TNF may cause marked tissue necrosis that is characteristic of progressive tuberculosis and may result in TNF release into the circulation, contributing to systemic manifestations of tuberculosis such as fever and cachexia.

Transforming growth factor (TGF-β) inhibits cytokine synthesis by macrophages and down regulates class II MHC expression. TGF- β also inhibits IL-2 dependent T cell proliferation and IL-2 receptor expression. TGF- β is produced constitutively by monocytes from tuberculosis patients and production is increased in response to PPD. Langhans giant cells and epethelelioid cells in tuberculous granulomas also express m RNA for TGF- β, suggesting that local production of TGF- β may result in deactivation of macrophages and immunopathology. Furthermore, IFN-y enhances antimycobacterial activity of macrophages only in presence of neutralizing antibodies to TGF- β. These results indicate that TGF- β inhibits antimycobacterial immune defenses and facilitates mycobacterial survival (Barnes and Modlin, 1996).

**3.7.5. CD3 lymphocyte and its activities.**

T cells are mainly confined to a region referred to as the paracortical ( or thymus dependent) area; in nodes taken from children with selective T cell deficiency, the paracortical region is seen to be virtually devoid of lymphocytes (Roitt, 1997).

The T cell receptor is responsible for the recognition of specific MHC antigen complexes and is different for every T cells. Thus after antigenic stimulation, each T cell or cloned formed by the expansion of T cell caries an α / β receptor of unique antigen specificity (Chakraborty, 2001).

In all immunocompetent T cells, T cell receptor none covalently but still intimately linked in a complex with CD3 molecule. CD3 molecule is believed to act as a single transducer that transduces antigen recognition signal to the interior of the cell received by α and β heterodimer. So far, more than 50 T cells antigens are identified by monoclonal antibodies.

CD3 antigen is present in all T cell and has a constant structure. It is closely associated with T cell receptor on cell membrane. CD3 molecule is made up of four noncovalently associated polypeptide chains (gamma, delta, eta and zeta) and is thought to be involved in transmitting signal to the interior of the cell following antigen binding (Roitt, 1997).

### 3.7.6. CD4 T lymphocyte and its Cytolytic activity:

An alternative mechanism by which T cells may contribute to immune defense is through direct cytolysis of macrophages and nonphagocytic cells infected with *Mycobacterium tuberculosis*. Kaufmann has suggested that many macrophages infected with *Mycobacterium tuberculosis* have low antimycobacterial potential allowing the bacilli to invade host defenses .Cytolytic T cells that specifically recognize mycobacterial antigens can lyse these macrophages with greater antimicrobial activity. Alternatively, cytolytic T cells may play a scavenger role by lysing dead macrophages containing large numbers of bacilli, so that they can be catabolised by surrounding mononuclear cells. Another hypothesis is that cytolytic T cells cause immunopathology by destroying infected macrophages, which in turn release toxic product that result in caseous necrosis (Barnes and Modlin, 1996).

### 3.7.7. CD8 T Lymphocytes:

$CD8^{+}$ T cell lines and clones lyses *M tuberculosis* primed macrophages in an antigen specific manner and restrict the growth of *M tuberculosis* in macrophages (Barnes and Modlin, 1996).

CD8 cells produce cytokines with a Th1 profile and function as MHC class1 cytotoxic T cells. This subset play a significant role in the expression of resistance and the principal role may be to lyse infected cells in lesions that still contain a few bacteria and thus sterilize the granulomas. CD8 cells seem to be of particular importance for the normal generation of protective granulomas and the lack of this subset leads to diffuse and badly organized cellular infiltrates (Khadka, 2000).

After T cell activation in presence of foreign antigen, it helps activation of B cells via Th2 (T helper) cells.

### 3.7.8. CD20 cells

The primary follicles of lymph nodes are composed of a homogenous cell population of small, darkly staining, unstimulated lymphocytes. The secondary follicles are those stimulated by antigens and include well developed germinal centers composed of paler staining heterogeneous

population of cells. These predominantly include B lymphocytes, small and large, cleaved and none cleaved as well as a few scattered T lymphocytes. The lymphocytes of the mantle zones are of B cell type. For immunophenotyping lymph nodes in paraffin embedded sections, reliable and identification of B lymphocytes can be achieved with the monoclonal antibody L26 directed against B cell marker CD20 (Ioachim, 1994).

For the identification of B cells, there is presently available in array of Monoclonal antibodies (MAbs) that detect various cellular markers. Most useful in the immunophenotyping of leukemias and lymphomas are those MAbs whose reactivity is restricted to B lymphocytes and there precursors. Such is antigen CD19, the most broadly expressed marker of B cells which appears at the earlier stages of B cell differentiation and persists throughout B cell maturation until the plasma cell stage, when it is no longer expressed. CD19 antigen has not been identified on normal or malignant T cells or myelomonocytic cell. CD20 antigen appears later in B cell maturation and persists until the plasma cell stage. But CD21 and CD22, B cell restricted antigens are expressed at late stages of maturation.

**3.7.9. S -100 protein:**

S 100 protein has been found in association with the interdigitating reticulum cells and dendritic reticulum cells. Interdigitating reticulum cells present the antigen to T cells where as dendritic reticulum cells present antigen to B cells. This in turn results in production of T helper cells and blastoid transformation respectively to overcome the disease (Ioachim, 1994).

**3.7.10. Co-operation between T and B cells:**

When T cells recognize and respond to carrier determinants, they help B lymphocytes specific for the hapten to develop into antibody forming cells, presumably by providing the required second or accessory signals (Roitt, 1997).

B cells produce antibodies, the production of which is enhanced by differentiation into plasma cells. Plasma cells are frequently found in tuberculous lesions. B cells (when activated) also increase the production of IFN-γ by NK cells and via antibody dependent cell mediated

cytotoxicity (NDCC); confer specificity to the killing of bacilli laden macrophages by NK cells (Dannenberg, 1999).

Antigenic stimulation in lymph nodes causes antigen-primed T and B cells to move in a synchronous fashion toward each other, meeting at the edges of B-cell follicles. These movements are orchestrated by dynamic changes in the expression of chemokine receptors. When CD4+ T cells are stimulated by antigen, they up-regulate the expression of two receptors (CXCR5 and CCR4) for chemokines produced in B-cell follicles; Th2 cells lose the ability to express CCR7 because chemokines that stimulate this receptor might otherwise keep them in the T-cell area. Conversely, antigen-stimulated B cells become responsive to macrophage inflammatory protein 3ß, which drives them toward the T-cell area (http://content.nejm.org/cgi/content/short/343/14/1020 2003).

**3.7.11. Mycobacterial antigens recognized by T lymphocytes**

*Mycobacterium tuberculosis* is a complex organism with a wide variety of protein antigens, which can be divided into structural and secreted antigens. Secreted antigens produced only by live organisms are likely to be the most important targets of T cells that confer protective immunity (Barnes and Modlin, 1996).

Of the wide variety of proteins secreted by *M tuberculosis*, three are of greatest interest from the standpoint potentially eliciting protective immunity in humans. Two such proteins are the 30 and 32 kDa members of the BCG 85 complex that are secreted in large quantities by rapidly growing mycobacteria. As these can bind fibronectin, a major component of human extra cellular matrix, they may mediate adhesion of bacilli to mucosal surfaces and perhaps subsequent intracellular invasion through macrophage fibronectin receptors. These proteins may therefore be critical virulence factor that are important targets of the immune response.

The 10kDa antigen, also referred to as BCG-a, is a secreted antigen that is associated with the cell wall of *M tuberculosis*. This antigen elicits more proliferation and IFN-y production by lymphocytes from healthy tuberculin reactors than do other culture filtrate antigens (Barnes and Modlin, 1996).

## 3.8. Hematologic changes during tuberculosis

Tuberculosis exerts a dazzling variety of hematological effects. These abnormalities involve both cell lines and plasma components (Oyre and Schlossberg,. 1999).

### 3.8.1. Red cells

Usually the anemia in tuberculosis results from anemia of chronic disease. In this situation, anemia is associated with a low or normal mean cell volume (MCV); this in turn reflects a shortened red blood cell (RBC) life span without a compensatory marrow response. Accompanying this change are decrease in serum iron, total iron binding capacity and transferrin saturation and an increase in serum ferritin, C reactive protein, and erythrocyte sedimentation rate (ESR). These abnormalities result from a redistribution of iron as an acute phage reaction.

### 3.9.2. Granulocytes

Chronic neutrophilia is well known and has occasionally prompted the search resulting in diagnosis of tuberculosis. Basophilia and eosinophilia are both described in tuberculosis. They also occur in patients with marked inflammatory response of other causes. Monocytes play an essential role in the immune response to tuberculous infection. True monocytosis is well documented as a consequence of chronic inflammation. There may be a significant quantitative increase in monocytes as well as morphologic changes. Circulating monocytes may be large and vacuolated and may in fact, be circulating macrophages. It is the monocyte/macrophage line that is responsible for the formation of granulomata.

### 3.8.3. Platelets.

Thrombocytosis is a well-known response to inflammation and is common in tuberculosis. The degree of thrombocytosis corresponds with the degree of inflammatory response as measured by the ESR.

### 3.8.4. Lymphocytes

Both lymphocytosis and lymphocytopenia are reported. Active tuberculosis causes a decrease in total T-cells secondary to a decrease in T4 cells. The CD4 count may fall transiently to fewer than 200/mm3, even in HIV negative patients. Total B-cells are also decreased. Multiple cytokines, including interleukin and tumor necrosis factor are activated.

## 3.9. HIV and Tuberculosis co-infection:

Clinical and epidemiological data indicate that persons co-infected with HIV and *M tuberculosis* are at increased risk for progressive disease from both pathogens. HIV infected patients are at markedly increased risk for the development of primary and reactivation tuberculosis (Small *et al.* 1994), and the manifestations of tuberculosis are more severe and life threatening in HIV infected persons (Barnes *et al.* 1991). Recent experimental data also indicate that co-infection with HIV and *Mycobacterium tuberculosis* results in significant alterations in the cellular immune response to both pathogens.

In mid 1980, the arrival of HIV supported tuberculosis to come back in the industrialized countries while TB was a major health problem in the developing countries. Today the co epidemic of TB and HIV is a major problem in the world. HIV increases the risk of getting TB thirty to fifty times. One third of the world's population has already been infected with TB and if these individuals contract HIV infection, it dramatically shortens their lives by causing an acute case of TB to erupt from their previously harmless infection. For someone who does not have a TB infection, but has contracted HIV, exposure to the TB germ can be devastating. The patients often die within weeks.

A healthy person who has been TB infected has less than a 10% lifetime chance of developing tuberculosis, where as an HIV infected person who is also infected with TB has up to 10% chance each year of developing a life threatening case of TB (STC/NTC, 2000).

## 3.10. Diagnosis:

Age, sex, socioeconomic status is the factors that contribute to the disease. And clinical features suggest the provisional diagnosis of tuberculous lymphadenitis. Chest radiograph, erythrocyte sedimentation rate, Mantoux test also provides the indirect evidence. For definitive diagnosis it requires the demonstration of *Mycobacterium tuberculosis* in culture or in AFB stain from the aspirated material. Histopatholoical examination and fine needle aspiration cytology also provides the diagnosis (Basnet, 1998). The most vulnerable groups are children who present with massive local lymphadenopathy even after mild infection. However, adult or elderly patients often react to infections with only slight to modest lymph node enlargement. (Thakur, Prakash *et al*.1998).

Infection probably follows minor dermal trauma or injury to the orphangeal mucosa. About 80% of patients have involvement of the cervical nodes. Specially, the sub mandibular & parotid groups. They may enlarge massively, causing a bull necks which interferes with breathing & swallowing. These nodes are not tender or painful & there is no pharangitis. There are no signs & symptoms of systemic disease. If untracked, chronic draining sinuses form. They cause permanent scarring when healed. Surgical excision nurses scorfula. Chemotherapy is not ordinarily used.

### 3.10.1. Clinical Feature:

Any of the cervical lymph nodes can be involved but anterior triangle of the neck is the frequent site. Lymph node swelling is painless and onset is insidious. They may be tender during the phage of rapid enlargement early in the infection. They feel rubbery, hard and matted due to periadenitis. Later on they suppurate to form abscesses and sinuses, and when the pus tracks down to the skin it leads to tuberculous dermatitis (scorfuloderma).

About two thirds of the patients present with the constitutional symptoms like low grade fever, weight loss and malaise. But the classical manifestations like fever, cough, failure to thrive or weight loss may not be found in the cases of tubercular lymphadenitis in clinical practice. Tubercular lymphadenopathy in immunosupressed patients may have a presentation resembling that of an acute pyogenic infection.

### 3.10.2. Radiological feature:

Radiological investigation is not very helpful in extrapulmonary tuberculosis is not variably confirmatory of tuberculous infection. Radiography examinations have little value as a general measure of case finding. Also that the short comings of radiography are

a. lack of definitiveness that is the mere presence of X- ray shadows is not indicative of a "case"
b. unless the presence of tubercle bacilli are demonstrated
c. high cost
d. High proportion of erroneous interpretation of films.

About 10-15% of culture positive tuberculosis on the basis of X-ray alone does not have tuberculosis. Therefore X-ray is neither sensitive nor specific for diagnosing and monitoring treatment of tuberculosis (Toman, 1979).

Thus mycobacterium containing tuberculostearic acid (TBSA) as a structural component of their cell walls and the detection of this substance in clinical specimens maybe used for the diagnosis of mycobacterial infections.

### 3.10.3. Laboratory diagnosis:

Previously *Mycobacterium bovis* was common for causing the lymphadenitis but nowadays the situation has changed and *Mycobacterium tuberculosis* is more common for causing tuberculous lymphadenitis (Basnet, 1998).

#### 3.10.3.1. Gross Appearance:

The gross features of tuberculosis are usually sufficiently distinctive to allow a tentative diagnosis to be made and to take precautions for avoiding infections. On section, the caseous necrotic area appears as creamy white patches, becoming chalky with the deposition of calcium. The periphery is densely fibrosed. In time, some nodules may be entirely converted into radiopaque, rock-hard masses (Stansfeld, 1985).

### 3.10.3.2. Microscopy

**Optical microscopy**

*Mycobacterium tuberculosis* is a thin rod with rounded extremities, 2-5um long and 0.2-0.3 um thick, non motile without capsule.

**Electron microscopy:**

Cell wall with 20 nm thickness and consists of an inner, electron dense layer surrounded by an outer, electron transparent layer no outer membrane covers outer layer of walls as is the case with gram negative bacteria (Bam, 2003).

### 3.10.3.3. Staining:

Different types of stains can be used for the detection of *Mycobacterium tuberculosis*. Stains can be classified according to specimens used for the organism detection.

**3.10.3.3.1. Z-N staining**:

Koch (1882) stained the tubercle bacillus with hot alkaline methyline blue as the primary stain and vesuvin as decolouriser and counterstain. Shortly afterwards Ehrlich(1882) discovered the now well known ‘acid fast’ property; staining the bacilli with hot fuschin in the presence of aniline oil as a mordant and destaining with the dilute mineral acid. Ziehl changed the mordant to phenol and Neelsen combined the dye and mordant to form carbol fuschin. Thus the staining technique although pioneered by Ehrlich, is now known as the Ziehl-Neelsen (ZN) method. ZN stain, hot stain is more preferable than the cold Kinyon stain (Grange, 1990).

The identification of characteristic beaded, rod shaped, acid fast bacilli is needed for positive diagnosis and can be made on the special, acid fast stains. Preferential locations are at the periphery of necrotic areas, among the epethlioid cells and rarely within the cytoplasm of giant cells. The number of bacilli in tuberculous lesions varies greatly depending on local immunity, age of the lesion and previous treatment. It has been estimated that minimum 10,000 organisms per mgm of tissue must be present to be identified by a special acid fast stain. The search for acid fast bacilli in paraffin embedded tissue is often disappointing, and not infrequently the search for acid fast bacilli in lesions morphologically typical for tuberculosis is unsuccessful. In such cases,

the diagnosis of tuberculosis should not be ruled out and attempts at confirmation must be pursued by other means (Ioachim, 1994).

**3.10.3.3.2. Fluorescent staining:**

The technique uses rhodamine- auramine stain for the detection of mycobacterial infection. Though it requires special microscope for its detection, it yields better identification of the bacilli. Also that lager number of samples can be observed in limited time without much stress.

**3.10.3.3.3. Haematoxylin-Eosin (H-E) Staining:**

Haematoxylin is the most widely used and versatile dye in histological technique and is used in stains for the demonstration of cell nuclei, myelin, elastic fibres, fibrin, neuroglia and muscle striations. For all these purposes however, haematoxylin must be used in conjunction with a mordant such as the salts of aluminum, iron or tungsten. Harri's haematoxylin a powerful and selective nuclear stain giving sharp delineation of nuclear structure. Eosin, a red dye, properly used on well fixed material, stains connective tissue and cytoplasm in varying intensity and shades of the primary colour giving a most useful differential stain. With haematoxylin, it is the routine stain in histopathology and much of the present knowledge of morbid histology has been gained from the study of H.E. stained sections.

In Haematoxylin-Eosin stain, presence of granulomas, classically described as having a centrum of necrosis and concentric areas of Langhans giant cells, epithelioid cells and lymphocytes is the diagnostic feature. More often, however the follicles are confluent, resulting in irregularly shaped areas of necrosis surrounded by epithelioid cells, occasional giant cells, lymphocytes, plasma cells and fibroblasts. The caseous necrosis characteristic of tuberculosis is a coagulative, total necrosis that leaves no cellular traces or nuclear debris. The giant cell of Langhans type has a strongly acidophilic cytoplasm and peripherally arranged multiple nuclei. This is not however always the case and multinucleate giant cells of various types can be observed (Ioachim, 1994).

**3.10.3.3.4. Immunohistochemical Staining:**

Immunocytology has been introduced to the study of pathology, and immunochemical methods have become routine procedures in the practice of histopathologic diagnosis. The identification of B cells, T cells, histiocytes and their subsets is feasible, depending on the recognition of characteristic cell markers. The development of highly specific Monoclonal antibodies that define the cell surface antigen made possible the identification of the various stages of B and T cell differentiation as well as detection of monoclonal cell populations (Ioachim, 1994).

**CD3 (T-cell marker):**

The CD3 molecule consists of five different polypeptide chains with molecular weights ranging from 16 to 28 kD. The five chains are designated gamma, delta, epsilon, zeta and eta. The CD3 complex is closely associated at the lymphocyte cell surface with the T cell antigen recepter (TCR). It is believed that the CD3 complex is involved in signal transduction to the T cell interior following antigen recognition. The CD3 antigen is first detectable in early thymocytes and its appearance probably represents one of the earliest sings of commitment to the T cell lineage.

**CD20 (B-cell marker):**

It is a non-glycosylated, four times membrane spanning protein with a molecular mass of approximately 33kDa in resting B cells. After mitogen stimulation it is heavily phosphorylated and appears as isoforms of molecular masses 33, 35 and 37 kDa. It is suggestive that phosphorylation may be a molecular mechanism involved in CD20 function.

**S-100 protein:**

S100 belongs to the family of calcium binding proteins such as calmodulin and troponin C. S100 a is composed of an alpha and beta chain whereas S100 b is composed of two beta chains. S100 protein is also expressed in the antigen presenting cells such as the Langerhans cells in skin and interdigitating reticulum cells in the paracortex of lymph nodes (http://www.neomarkers.com 2003).

### 3.10.3.4. Culture:

Robert Koch originally grew the tubercle bacillus on heat coated bovine or sheep serum- a culture medium invented by the Irish physicist John Tyndall. Common is Lowenstein Jensen media containing egg, glycerol, asparagines, mineral salts and malachite green dye; the dye inhibits certain contaminating bacteria in primary culture and provides a green background for the better visualization. Similarly, Ogawa medium containing egg yolk, instead of whole egg but other media, Stonebrinks medium also can be used; Sauton's medium can also be used for the cultivation of mycobacteria for the immunological studies. But when grown in liquid media, unless a detergent such as Tween 80 is included in the medium, the hydrophobic nature of the lipid cell wall causes the bacilli to grow as fungus- like surface pellicles. Especially strains of *M tuberculosis* produce characteristic serpentine cords. It can be cultured in: Semi synthetic agar media, Inspissated egg media, Broth media, etc. (Grange, 1990).

### 3.10.3.5. Serology:

Use of serum for the detection of different antigens of *M tuberculosis* can be done by different ELISA techniques. Detection of PPD and Lipoarabinomannon antigens of *M tuberculosis* can also be done. In the literature, the sensitivity and specificity of serological tests have been reported to be 70-80% and 90-95%, respectively. Results tend to depend on the nature of antigens, testing formats and the prevalence of TB among general population on the study areas. Due to chronic nature of TB, however it is difficult to decide whether the disease is current based on the presence of elevated antibodies to *M tuberculosis* antigens. (Cho, Chi *et al*. 2001).

### 3.10.3.6. Erythrocyte sedimentation rate (ESR):

Erythrocyte sedimentation rate is a nonspecific test; because it can be raised in any chronic diseases. Its measurement is useful index during follow-up. Most of the study shows raised ESR in over 90% of the cases (Baskota, Pradhan, *et al*. 1995).

### 3.10.3.7. Tuberculin (Mantoux):

Also called as tuberculin test is one of a most widely used tool for the diagnosis of tuberculosis. It is a delayed type hypersensitivity reaction. When we inject the tuberculo

protein the initial influx at four hours are the neutrophils but after 24 hours infiltration of monocytes and T-cells occur. In this reaction, CD4 T cells outnumber CD8 cells by 2:1. CD1 T cells are found in the dermal infiltrate. Monocytes constitute 85%-90% of the total cellular infiltrate. Macrophages are probably the main antigen presenting cells in the tuberculin hypersensitivity reaction (Hashino, 1996).

Positive tuberculin skin test (Mantoux test) indicates the tuberculous infection in past, present or BCG vaccination. Predictive value of Mantoux test was 100% in one study done in Hong Kong. In other study they found that Mantoux test was positive in 98.3%. But the conditions which may suppress the tuberculin skin test are HIV infection, malnutrition, severe bacterial infections including tuberculosis itself (miliary tuberculosis) viral infections for example: meseals, chickenpox, glandular fever, cancer, immunosuppressive drugs like steroids (WHO, 1996).

#### 3.10.3.8. Polymerase chain reaction (PCR):

This is a new technique in which we can amplify the DNA sequences using a proper DNA probe. PCR is the most sensitive technique in the demonstration of *Mycobacterium tuberculosis* in clinically suspected patients, who have AFB stain or culture negative. The PCR sensitivity is 80-85% with a specificity of 99% (Thomas, 1994).

#### 3.10.3.9. Chromatographic Identification:

The gas liquid chromatography (GLC) (MIDI, Newark, DE) procedure provides a rapid preliminary and sometimes definitive identification of cultures. High performance liquid chromatography (HPLC) is currently advocated by many laboratories (Zheng and Roberts, 1999).

## 3.11. Treatment:

### 3.11.1. Chemotherapy:

Tuberculosis is 100% curable disease. Selman A Waksman and his colleagues, working in the USA discovered streptomycin, the first effective antibiotic drug against Tuberculosis in 1994 (STC/NTC, 2000).

Treatment short course, which is a new strategy to control TB by giving drugs to patients under direct observation of health workers. DOTS has been found 100% effective to cure TB and to prevent multi drug resistance. Only DOTS ensure cure of diagnosed TB patients. It can also prevent relapse and death. Effective treatment of TB can prolong survival of patients with AIDS. Through the general health services it can be used widely. The global targets for TB control are to cure 85% of new sputum smear positive cases and to detect 70% of such cases. DOTS strategy has achieved these results on programmed basis in our region (STC, 2000).

The eradication of Tubercle bacilli by chemotherapy may be considered in 3 stages: first, the destruction of large number of extra cellular bacilli in the walls of cavities: second, the killing of the smaller population of least actively growing bacilli within macrophages and necrotic tissue; and the third, the elimination of an even small number of dormant (or near dormant) bacilli within macrophages or dense caseous material. When superficial lymphadenitis is detected before extensive caseation, periadenitis, or erosion has occurred, chemotherapy is nearly always curative (Schlossberg, 1999).

**Isoniazid**: Isoniazid results in loss of acid fastness, probably as a result of the inhibition of the synthesis of mycolic acids (Heym, Philip *et al*. 1996). Isoniazid rapidly kills bacilli in the walls of the cavities, leading to a sharp drop in the numbers of viable bacilli in the sputum; it is less effective against slowly replicating bacilli. Streptomycin also destroys extra cellular bacilli, but only if they are in an alkaline environment.

**Ethambutol**: Ethambutol has some bactericidal activity in the early stages of chemotherapy but is not a sterilizing drug and accordingly, is usually given during the first two months of treatment. It is no better than streptomycin in preventing the emergence of drug resistant but has the advantage that it may be given orally (Grange, 1990).

**Rifampin**: Rifampin, a lipophilic ansamycin, is highly active against mycobacteria as it diffuses rapidly across the hydrophobic cell envelope. This is the key component of anti tuberculous

therapeutic regimens and its use has greatly shortened the duration of chemotherapy necessary for the successful treatment of drug susceptible tuberculosis (Heym, Philip *et al*. 1996).

**Pyrazinamide**: Pyrazinamide due to its powerful activity in an acidic environment it was proposed that pyrazinamide should be particularly active on intracellular bacilli or in recent caseous lesions (Heym, Philip *et al*. 1996).

In a research conducted by Behera and. Mallik demonstrated that the same medicine isoniazid, rifampicin and ethambutol can be given to cure tuberculous lymphadenitis. These drugs given on daily basis for 36 weeks resulted in complete resolution of lymph nodes in 65.7% of the tuberculous lymphadenitis cases.

**3.11.1. a. Multi-drug resistance (MDR) of *M tuberculosis*:**

Several recent studies indicate that resistance to various anti-tuberculous agent results from alternations to chromosomal genes encoding the drug targets. Thus, multidrug resistance does not stem from the acquisition of *Mycobacterium tuberculosis* of a transposable element, or a plasmid, carrying drug resistance determinants, but rather appears to result from the stepwise acquisition of new mutations in the genes for different drug targets. A number of operational difficulties like inadequate prescription of chemotherapy, poor compliance or an insufficient number of active drugs in the regimen may be responsible for their selection (Heym, Philipp, *et al*., 1996).

As postulated many years ago, it appears that random mutations conferring resistance occurs naturally during microbial replication (Canetti and Grosset, 1991). These mutations arise independently and are not induced by drugs but strains harboring them maybe selected by incorrect drug use (Small *et al.* 1993).

MDR is a potential obstacle to the successful treatment of tuberculosis. Where ever MDR is common; it shows the poor performance of TB control programe. Treatment failure rates are high in MDR endemic areas. The resistance level in a given population can be reduced by the implementation of sound TB control policies and DOTS (STC, 2000).

### 3.11.2. Excision biopsy:

Management of tuberculous lymphadenitis involves appropriate use of antituberculous chemotherapy with the judicious use of surgical excision in a minority of patients. Total excisional biopsy should be performed because an incomplete biopsy nearly always results in ulcerations or sinus tract formation (Powell, 1999). If not done for diagnostic purposes, the surgery should be limited to those patients who fail to show improvements after an adequate course of chemotherapy or who have discomfort from enlarged or tense, fluctuant nodes (Campbell, 1990).

## 3.12. Vaccine:

**Bacillus Calmette Guerin (BCG):**

Suppurative lymphadenitis following BCG vaccination is usually self-limiting and the reported incidences are 0.1 to 4%. Outbreaks of suppurative lymphadenitis following BCG vaccination have been reported from various countries. Most of the times these outbreaks have occurred after a change of strain or the source of manufacturer. BCG vaccination does not prevent natural tuberculosis infections of lung and its local complications, though it reduces the incidence of haematogenous complications.

Various therapeutic modalities of chemotherapy, placebo therapy, complete surgical excision and needle aspiration had been tried in the management of suppurative lymphadenitis. There is strong evidence that chemotherapy does not have any role in achieving the regression of the lymph nodes. Surgical excision was considered by some workers to be the ideal therapeutic modality. Needle aspiration is of immense value in the diagnosis and management of this entity. Medical treatment may not be required and repeated aspiration of the contents suffices to achieve complete resolution (Satayanarayana S, 2002).

The WHO suggests that drainage and direct installation of an antituberculosis drug into the lesion be considered for adherent or fistulated lymph glands. Lesions that are non adherent will heal spontaneously without treatment. Systemic treatment with antituberculosis drugs is ineffective (WHO,1986).

# 4. MATERIALS AND METHODS

## 4.1. Materials and chemicals used:

1. Avidine-Biotin complex reagent
2. Primary antibodies of CD5 T-cell marker, (Novo Castra Laboratory)
3. Primary antibodies of CD20 B- cell marker, (Dako Corporation)
4. Primary antibodies of S-100 Histocyte marker, (Neomarkers)
5. Formalin; Qualigens, Glaxo India Ltd.,India.
6. Ethanol; Qualigens, Glaxo India Ltd.,India.
7. Methanol Qualigens, Glaxo India Ltd.,India.
8. Xylene Qualigens, Glaxo India Ltd.,India.
9. DPH; Qualigens, Glaxo India Ltd.,India.
10. Paraffin Wax; Qualigens, Glaxo India Ltd.,India.
11. AFB staining reagents.

    Carbol fuschin: Qualigens, Glaxo India Ltd.,India

    Phenol: Qualigens, Glaxo India Ltd.,India.

    Absolute alcohol: Qualigens, Glaxo India Ltd.,India.

    Methylene blue : Qualigens, Glaxo India Ltd.,India

    Acitic acid: Qualigens, Glaxo India Ltd.,India.

    Hydrochloric acid: Qualigens, Glaxo India Ltd.,India.

Alcohol (95%) Bangal Chemicals, India.

12. H - E staining reagents

Haematoxylin: Qualigens, Glaxo India Ltd.,India
Absolute alcohol: Qualigens, Glaxo India Ltd.,India.

Ammonium or potassium alum: Qualigens, Glaxo India Ltd.,India

Mercuric oxide: Qualigens, Glaxo India Ltd.,India.

**Others:**

1. Specimen (Lymph node)
2. Auto Technicon; Technicon Corporation, New York, U.S.A
3. Microtome “820” spencer; AO Company, U.S.A
4. Microtome Blades; Feather, Japan.
5. L- shaped key
6. Water bath
7. Refrigerator

## 4.2. Collection and Storage of Specimens:

Lymph node biopsy were collected from the patients attending OPD at Patan Hospital during the year 2058-2059 with chief complaint of fever, lymph node pain, loss of weight or loss of appetite. Patients may or may not have palpable lymphadenopathy as clinical presentation. Clinical diagnosis of tuberculous lymphadenitis and lymph node biopsy for the confirmatory diagnosis was referred by attending physicians.

Excised lymph nodes were preserved in 10% formalin solution till processing. All the specimens were processed within 2 days of collection.

## 4.3. Processing of Specimens:

The stored specimens were then embedded and sections were cut from it.

### 4.3.1. Embedding.

The lymph node biopsy kept in formalin solution was first cut, the process called as gross cutting. The specimen thus prepared was placed in auto-technicon machine which was set to operate overnight. After the completion of process in auto-technicon, the specimens were taken out labeled and blocks were prepared by embedding in paraffin wax (DAKO, 2001).

### 4.3.2. Section cutting:

First, the blocks were cut roughly to expose the specimen and then were stored in Deep fridge.Then, fine cutting of the sections (3-5um thin) were done by adjusting the knob as desired. Section thus cut was first placed in warm water for few seconds and was attached to dry slide and allowed to dry. Thus prepared slides were processed for ZN stains and Immunohistochemical stains as below (DAKO, 2001).

## 4.4. Pre Staining:

Removal of embedding media exposes the specimen impregnated called as deparaffinization. This after the addition of different stain using standard protocol retains the colour.

#### 4.4.1. Deparaffinization:

Prior to staining, tissue slides were deparaffinized to remove embedding media and then rehydrated as per standard protocol (DAKO LSAB2 System, Peroxidase).

1. The slides were first placed in hot air oven for 5 minutes at $60^0$c.
2. The slides were then placed in a xyline bath and were incubated for 5 (±1) minutes. Baths were changed and the process was repeated once.
3. The slides were then placed in absolute ethanol for another 3 (±1) minutes. Baths were changed and the process was repeated once.
4. The slides were then placed in 95% ethanol for 3 (±1) minutes. Baths were changed and the process was repeated once.
5. The slides thus prepared were placed in distilled water for 1 minute.

## 4.5. Staining methods:

After deparaffinization and rehydration of the specimen section, they were stained as desired.

#### 4.5.1. Z-N staining method:

Ziehl-Neelson staining for *Mycobacterium* spp. of the samples scrapped from the biopsy samples and their microscopic observation (1882-1883); Techniques for *Mycobacteria.*

1. Deparaffinized and rehydrated specimens in the slides were flooded with the filtered carbol fuschin.
2. The slides were heated to steaming with intermittent flaming for 15 minutes.
3. The slides were washed well in tap water.
4. Slides were then decolorized by 1% acid alcohol for 1 minute.
5. Again the slides were washed well in tap water.
6. Smear was counter stained with 0.25% acidified methylene blue for 30 seconds to 1 minute and was washed with distilled water.

7. Slides were allowed to dry, then dipped in xyline solution and mounted with DPH.
8. Slides were observed in microscope at 100X under oil-immersion.

**4.5.2. Haematoxylin and Eosin Staining:**

Harris' Haematoxylin method and Eosin stain was used for staining histological sections. (Harris, 1900).

1. Deparaffinized and rehydrated specimens in the slides were flooded with the filtered Harris' Hematoxylin in a jar for 5 minutes.
2. The slides were washed well in running tap water for 2-3 minutes.
3. Then it was decolorized with 1% acid alcohol.
4. The decolorisation was stopped by washing it with alkaline water for 5 minutes.
5. Slides were than flooded with 1% aqueous eosin and were left for 2 minutes.
6. Again the slides were washed in running tap water for 5 minutes.
7. Slides were allowed to dry, then dipped in xylems solution and mounted with DPH.
8. Slides were observed in microscope at 5X, 10X and 40X respectively under oil-immersion.

**4.5.3. Immunochemical Staining:**

All three immunochemical staining were done by two step method using mouse monoclonal antibody as primary antibody diluted 1:100 folds. The process was done by standard ABC (avidine-biotin complex) technique. The immunohistochemical staining was done at a collaborating centre at Seoul, South Korea.

Briefly the procedure was as follow:

1. Slides were gently rinsed with distilled water and were placed in buffer bath for 5 mins.
2. Excess liquid was removed from around the section.

3. 4-6 drops of rabbit serum, diluted 1:5 - 1:20 was added and incubated for 20-30 mins.
4. Serum was tapped off and excess was wiped away.
5. 4-6 drops of mouse primary antibody was added and was incubated for 20-30 min.
6. Step 1 and 2 were repeated.
7. 4-6 drops of approximately diluted biotinylated rabbit anti-mouse antibody was added and incubated for 20-30 mins.
8. Step 1 and 2 were repeated.
9. 4-6 drops of Avidin-Biotin complex (mixed and diluted approximately at keast 30 mins before use) was applied and was Incubate for 20-30 mins.
10. Steps 1 and 2 were repeated.
11. Substrate- chromagen solution was applied and was incubated until desired color intensity developed.
12. Slides were rinsed gently with distilled water from wash bottle and were counter counterstain.

# 5. RESULT

All the ZN, Haematoxylin-Eosin and immunohistochemical stained slides were observed processed and observed as per standard protocol mentioned below. AFB Stain: method. H-E

The results were noted in result sections.

## 5.1. Distribution of tuberculous lymphadenitis in different age and sex groups.

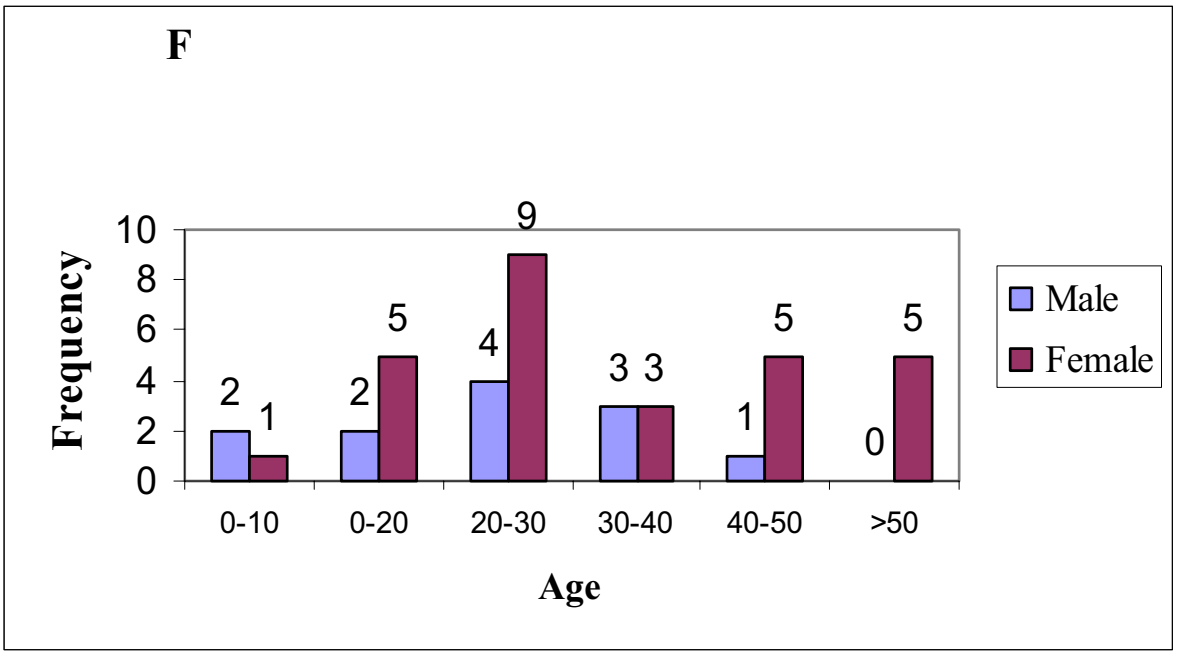



**Fig 5.1 Age and sex wise distribution of tuberculosis lymphadenitis**

Among of 40 suspected cases of tuberculous lymphadenitis, male (30%) were lower than female (70%). Higher prevalence was found in patients of 20-30 years of age group. The cases were observed in age group 1 to 55 years.

## 5.2. Site and number of lymph nodes involved in tuberculous lymphadenitis.

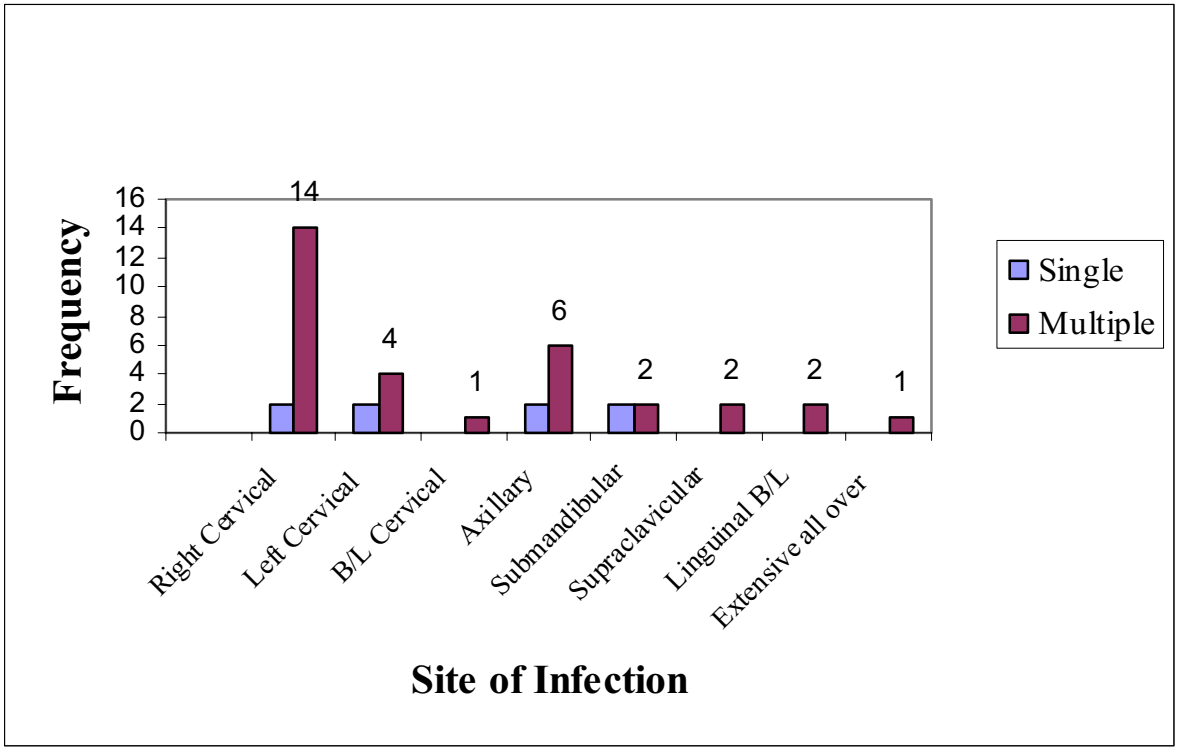
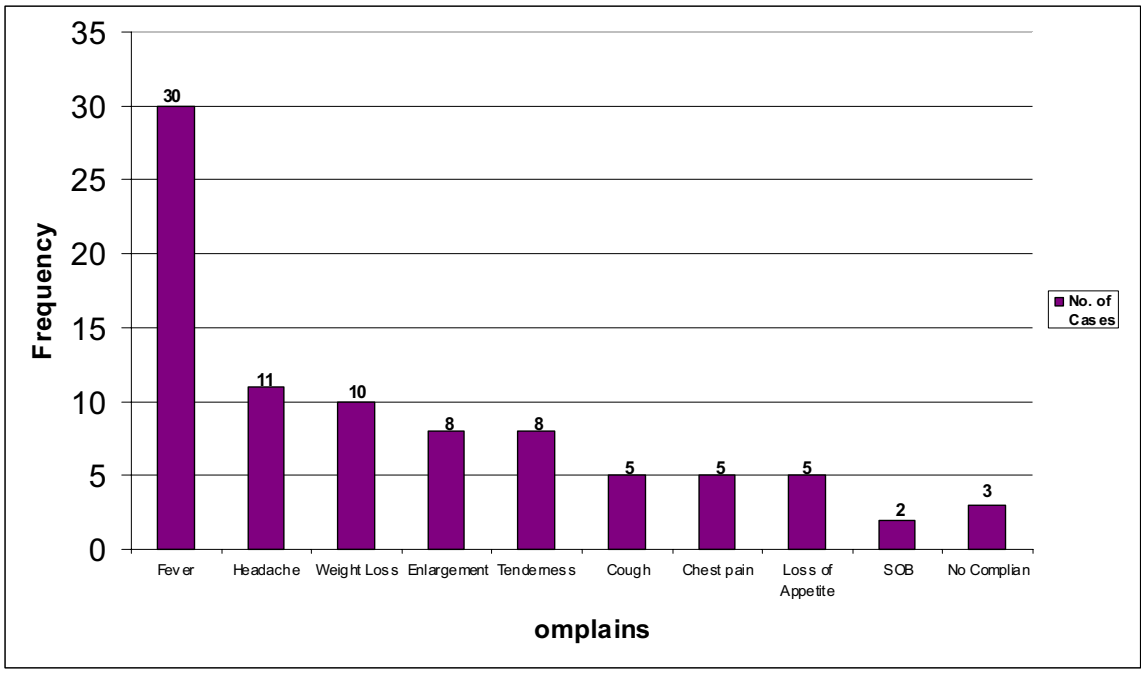



**Fig 5.2. Site and Number of Lymph nodes Involved in Tuberculous Lymphadenitis**

Cervical lymph nodes were the most common lymph node involved in tuberculous lymphadenitis, which covers about 57.5% of the total 40 cases. Right (40%), left (15%) and bilateral (2.5%) cervical lymph nodes involvement were seen. Axillary lymph nodes (20%), submandibular lymph node (10%), Supraclavicular (5%), Lingual bilateral (5%) and a single case of extensive lymph node (2.5) were involved. Multiple lymph node infection was found in 80 % of the cases where as single lymph node involvement was found in only 20% of the cases.

## 5.3. Co-relation of tuberculous lymphadenitis with sign and symptoms.




In 75% of the cases, patients had chief complaint of fever where as headache and weight loss was observed in only 27.5% and 25% of the cases respectively. Enlargement & tenderness of the lymph nodes were observed in 20% of the cases each. Cough chest pain & loss of appetite was also complaint in 12.5% of the cases where as 5% had complaint of shortness of breath and 7.5% of the cases had no obvious complain.

## 5.4. Co-relation of AFB positivity and tuberculous lymphadenitis.

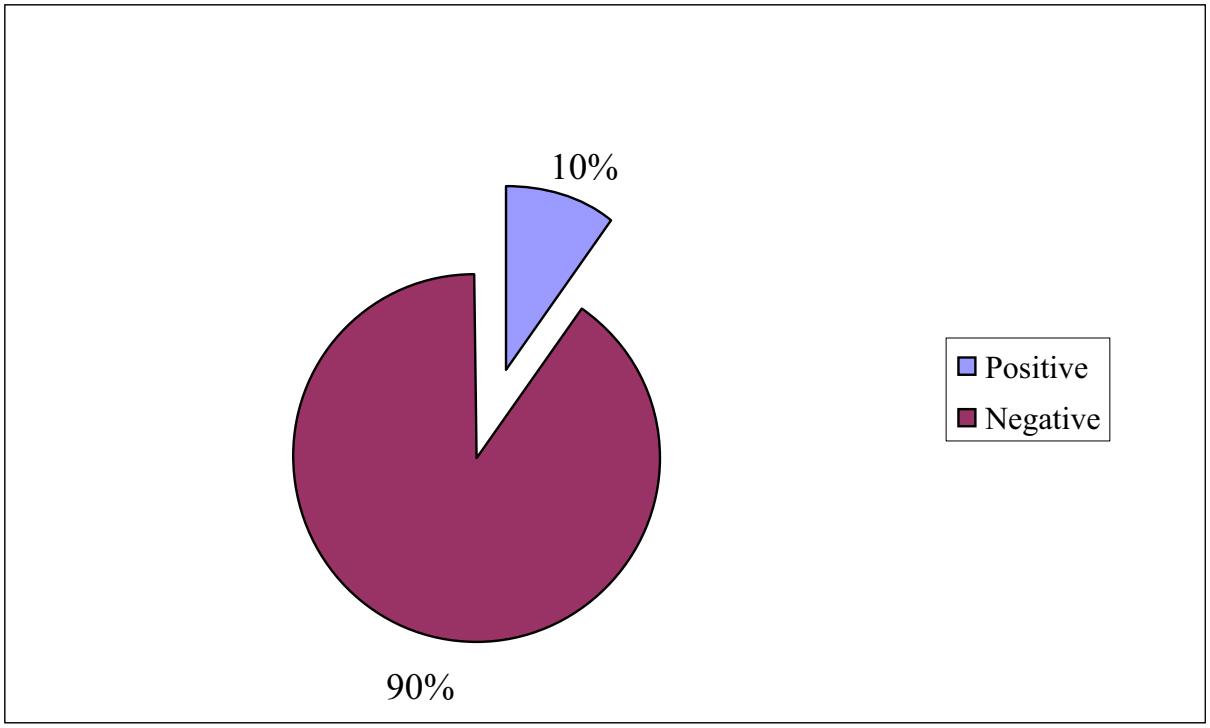



**Fig 5.4 Co-relation of AFB positivity and tuberculous lymphadenitis.**

Among 40 suspected cases of Tuberculous lymphadenitis, only 10% of the cases were found to be positive for Acid Fast Stain. Higher percentage of female was found to be infected than male percentage in AFB stain. In comparison to 90% of the female infected cases, only 10% of the male were infected cases were identified as positive of tuberculous lymphadenitis by AFB stain.

## 5.5. Co-relation of Haematoxylin-Eosin stain positivity and tuberculous lymphadenitis.

All 40 suspected cases of tuberculous lymphadenitis were found positive for tuberculosis in Hand E stain that is 100% positivity was observed in Haematoxylin-Eosin stain taking presence of granulomas and caseous necrosis as the diagnostic criteria.

## Table5.1. Giant cells and tuberculous lymphadenitis.

| Giant cell (No) | Frequency | Percentage |
|---|---|---|
| 0 | 2 | 5 |
| <5 | 18 | 45 |
| <10 | 9 | 22.5 |
| <15 | 5 | 12.5 |
| >20 | 6 | 15 |
| **Total** | **40** | **100** |

Giant cells were present in 95% of the total 40 suspected cases of tuberculous lymphadenitis cases. Where as, in 5% of the cases, no giant cells were found. Maximum percentage of the cases I.e. 45% had 1 to 5 numbers of giant cells followed by 22.5% of the cases with 5 to 10 numbers of giant cells and 12.5% had 10-15 number of giant cells. And the rest of the 15% cases had higher than 20 giant cells present.

**Table 5.2. Granuloma in tuberculous lymph node.**

| Granuloma (%) | Frequency | Percentage |
|---|---|---|
| 0-20 | 9 | 22.5 |
| 20-40 | 14 | 35 |
| 40-60 | 5 | 12.5 |
| 60-80 | 9 | 22.5 |
| >80 | 3 | 7.5 |
| **Total** | **40** | **100** |

In 35% of the total 40 suspected tuberculous lymphadenitis cases, 20-40 % of the cellular area was in a form of granuloma, followed by 22.5% of the cases with 0-20 and 60-80 % of the cellular area as granuloma. In 12.5 % of the cases, 40-60% of the cellular area was with granuloma and in only 7.5% of the cases, 80% of the area covered with granulomas.

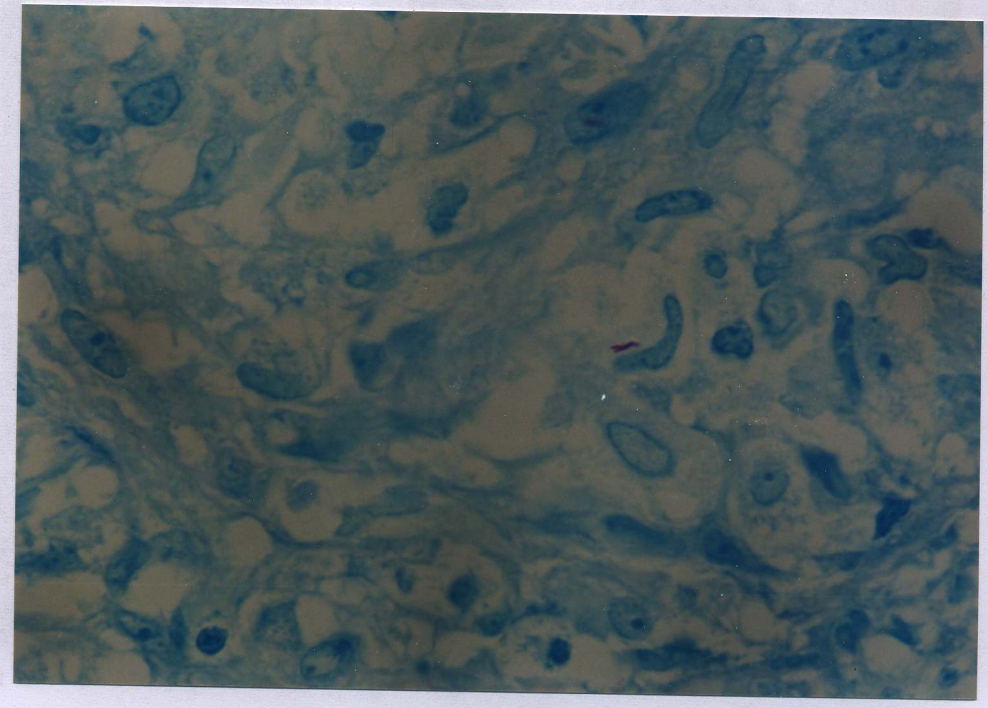
**Table 5.3. Cell Necrosis in tuberculous lymph node.**

| Necrosis (%) | Frequency | Percentage |
|---|---|---|
| 0-20 | 15 | 37.5 |
| 20-40 | 12 | 30 |
| 40-60 | 2 | 5 |
| 60-80 | 6 | 15 |
| >80 | 5 | 12.5 |
| **Total** | **40** | **100** |

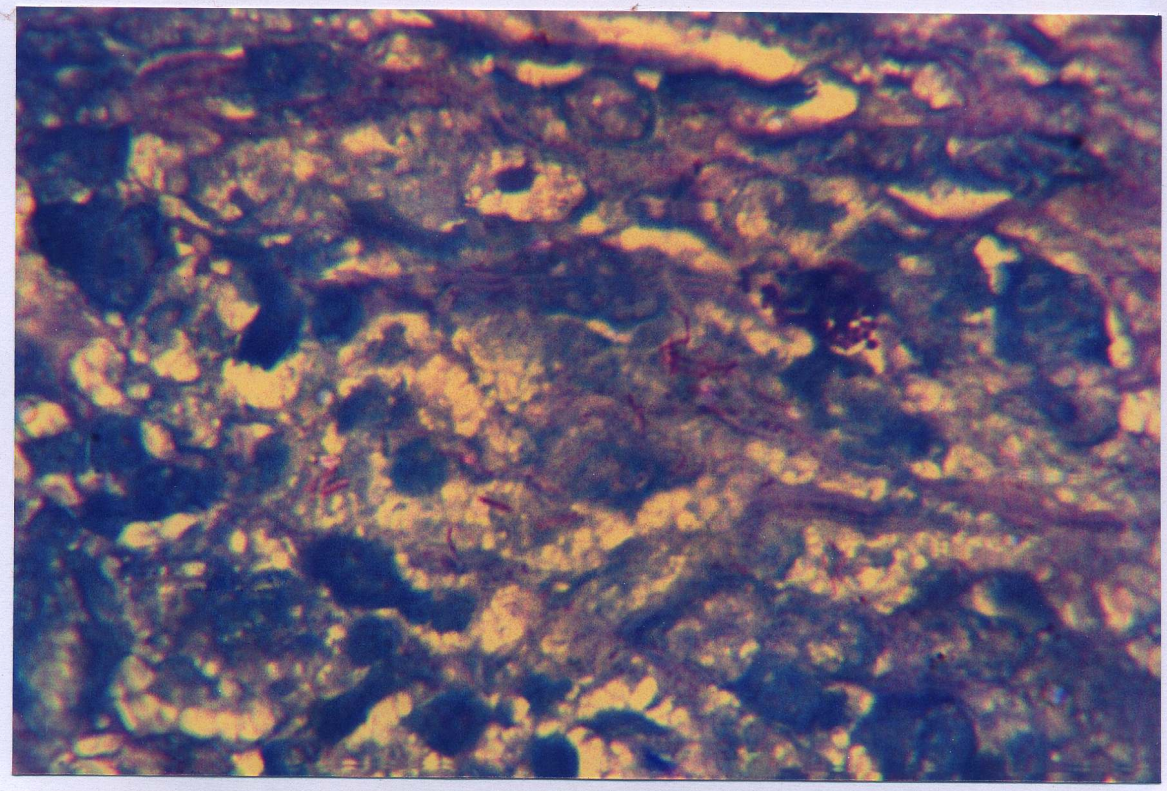
No cellular area of caseous necrosis was observed in all 40 suspected cases of tuberculous lymphadenitis. 35% of the cases had least necrotic area in which only 0-20% of the cellular area had necrosis. Similarly, 30% of the cases had 20-40% of the area with necrosis, 5% cases had 40-60% of the area with necrosis, and 15% of the cases had 60-80% of the area with necrosis. greater than 80% of the necrosis area was seen in only 12% of the cases.

## Table 5.4. Results of immunohistochemical stain.

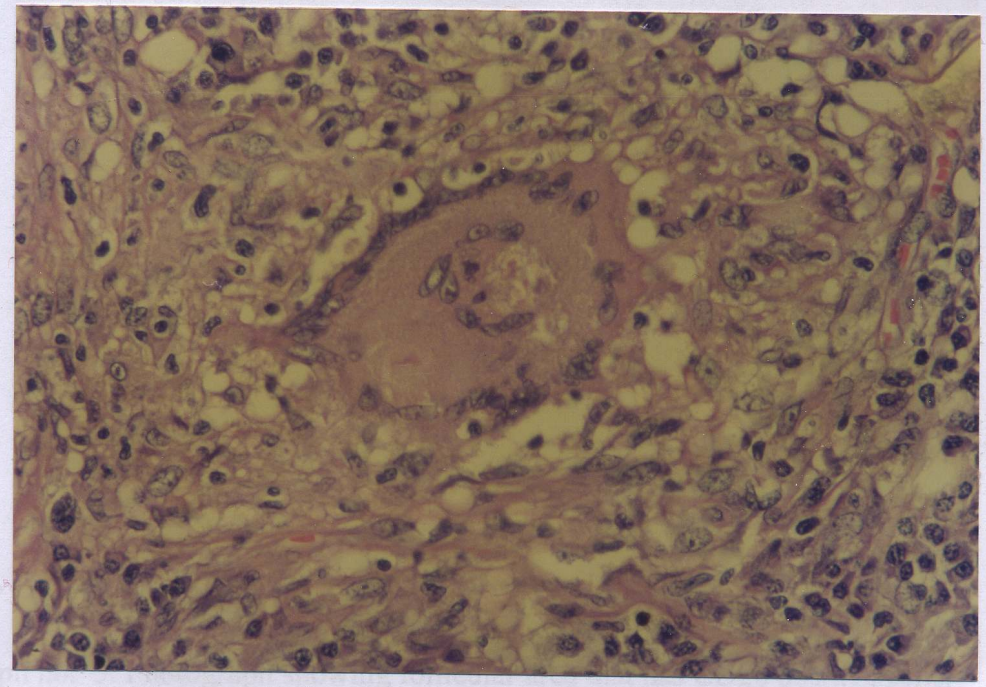
| **Stain Used** | **Cells stained** | **Part of lymph node** | **Observation** |
|---|---|---|---|
| CD3 | T cells | Paracortex | Positive/ Stained (+) |
| | | Follicular Area | Negative/Positive Unstained/Stained(±) |
| | | Interfollicular area | Positive/ Stained (+) |
| | | Langerhan's Giant cell | Negative/ Unstained (-) |
| | | Granuloma | Positive/ Stained (+) |
| CD20 | B cells | Paracortex | Negative/ Unstained (-) |
| | | Follicular Area | Positive/ Stained (+) |
| | | Interfollicular area | Negative/ Unstained (-) |
| | | Langerhan's Giant cell | Negative/ Unstained (-) |
| | | Granuloma | Negative/ Unstained (-) |
| S-100 | Dendritic Reticulum cells | Germinal Centres | Negative/ Unstained (-) |
| | Interdigitating Reticulum cells | Paracortex area | Positive/ Stained (+) |



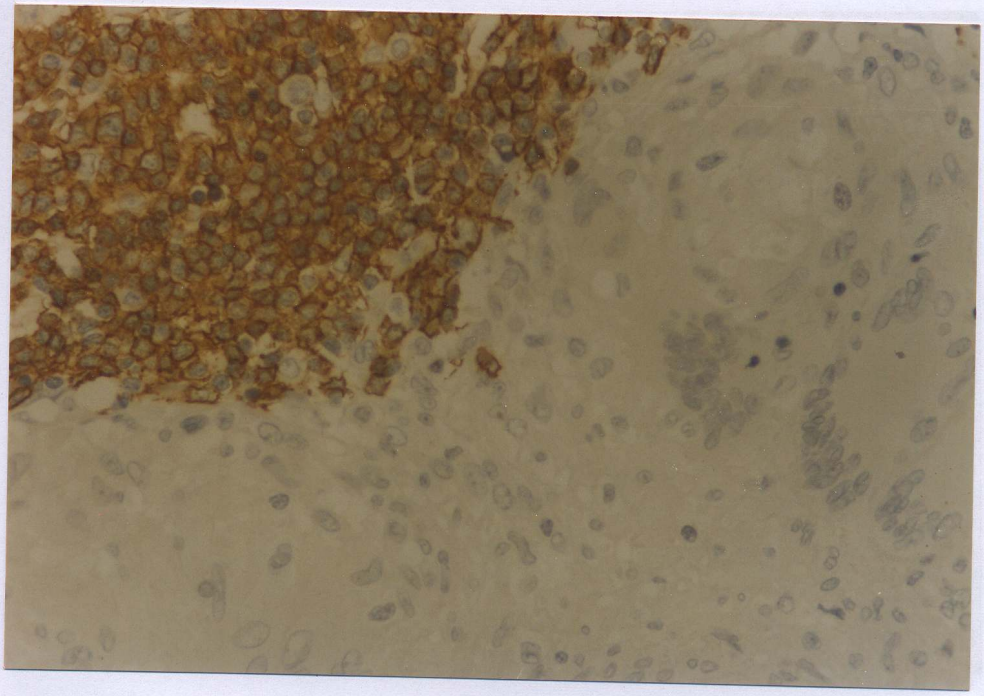
Photograph no. 1. Photograph showing two Acid Fast Bacilli on lymph node section stained with Ziehl-Neelson stain.



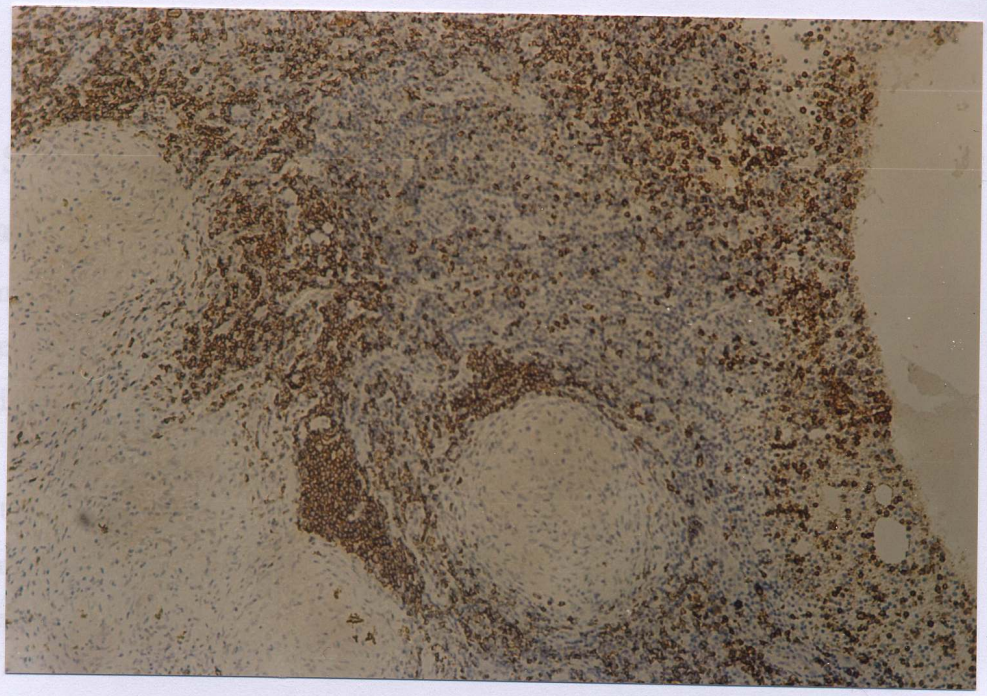
Photograph no. 2. Photograph showing bunch of acid fast bacilli on lymph node section stained with Ziehl-Neelson stain.



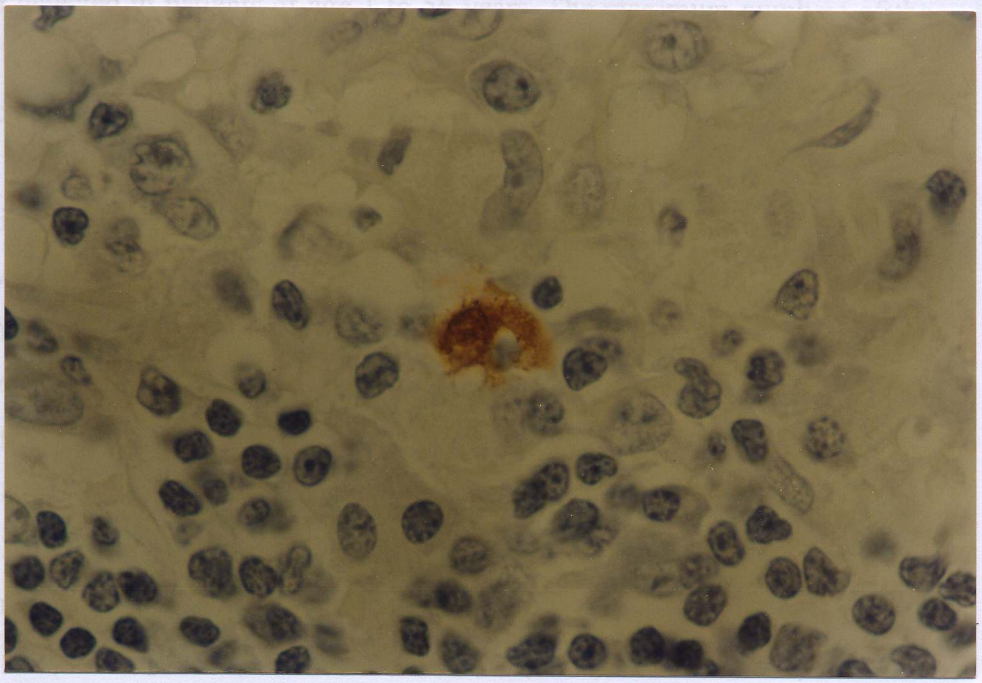
Photograph no. 3. Photograph showing Langhan's giant cell in Haematoxylin eosin stain.



Photograph no. 4. Photograph showing paracortical area that has CD3 positive cells.



Photograph no. 5. Photograph showing follicular area that has CD20 positive cells.



Photograph no. 6. Photograph showing lymph node section stained with S-100 positive cells.

# 6. DISSCUSSION AND CONCLUSION

## 6.1. Discussion

Tuberculosis being a communicable disease has been creating a lot of attention in Nepal; Lymph node tuberculosis has been one of the difficult in diagnosis since long. Preliminary diagnosis is based on certain clinical sign and symptoms which may vary with the individuals.

Various staining techniques including, AFB staining, Haematoxylin-Eosin staining in 40 and Immunohistochemical staining was performed in 20 biopsy samples collected at Patan Hospital for bacteriological, histological and immunological study.

The maximum numbers of patients infected were found to be female (70%) and then male (30%). Similar study conducted by Huhti, Brander, *et al.* (1975), in adult patients also had female predominance, however Basnet (1998), found 50.4% male cases and 49.6 % female cases.

The disease could be detected in patients of age 1 to 56 years. The maximum numbers of the cases detected were between the ages of 20-30, which is in agreement with a study done by Shrestha (1989) although principally lymph node tuberculosis should be higher in children.

Involvement of cervical lymph nodes was found in maximum number of cases i, e.57.5%. Of which, right node was involved in 40%, left node in 15% and 2.5%of the cases involved bilateral cervical lymph nodes. This finding was in agreement with findings of the studies done by Huhti, Brander, *et al.* (1975). The cause for higher rate of right sided infection than left sided might be due to wider, shorter and vertical right sided bronchus. Axillary lymph nodes were found involved in 20% of the cases, submandibular in 10%, supraclavicular in 5%, inguinal in 5% and extensive in 2.5% of the cases. Principally, one or more of the lymph node sites may be involved in infection by tubercle bacilli depending upon the immunity of an individual. The study agrees with a study conducted by Seth, Kabra, *et al.* (1995), with involvement of 80% cervical, 14% axillary and 7% inguinal lymph nodes respectively.

Single and multiple both types of node infection were seen. But in 80% of the cases, multiple nodes were found to be affected which was higher than 20% of the cases that involved single node which is similar to the findings of Basnet (1998) and Seth, Kabra, *et al*. (1995).

In, clinical symptoms majority of cases (75%) complained of fever with or without headache. Headache was found in 27.5% of the cases. Enlargement and tenderness of the lymph node was found in 20% of the cases. Chest pain, Cough and loss of appetite have been observed in 12.5% of the cases each. 7.5% of the cases were presented with shortness of breadth, since they were AFB negative in sputum smears; the possibility of pulmonary tuberculosis has been excluded. 5% of the cases had no complain at all where as 2.5% were presented with symptoms of nausea. This finding is similar to the similar study of Powell (1999).

Only 10% slides were found positive for AFB stain, which is higher than the finding of shrestha (1989). In which, only two cases of AFB positive slides out of 349 slides could be detected. Detection of low percentage of tubercle bacilli in lymph nodes may be due to reasons like, specimen sections of 3-5um in which, organisms in only one plane can be observed. Absence of the Mycobacterium in slides may be due to the fact that the bacillus lies within the macrophages, which could not be stained. But the slide which had unusually the higher number of bacilli could be detected which may be due to the patient being in an immuno-compromised situation like in HIV infection.

Among 40 clinical cases of tuberculous lymphadenitis, in all cases, epitheloid cell granuloma along with caseous necrosis and with or without giant cells in Haematoxylin-Eosin stain could be observed, which was considered as the diagnostic criteria for tuberculosis. In multinucleated giant cell, the nuclei were seen as blue coloured where as the cytoplasm as pink colour. Other cells were seen as blue colour in pink background. Present study shows that in 45% of the biopsy specimen only 0-5 numbers of giant cells were observed where as in 27.5% of the specimen had 5-10 numbers of giant cells, 12.5% had 10-15 numbers of giant cells and 15% had more than 20 numbers of giant cells. The data shows that majority of the cases had a frequency of giant cells between 0-5 in numbers.

Granuloma made up of epitheloid cells with or without central necrosis and giant cells are the results of active immunity of the individual. The size of granulomas cannot be determined since its size varies with the individual, roughly it has size of 1-2 mm. granuloma formation is the result of cell mediated immunity after the interaction with intracellular pathogens. But in patients with low immunity like in HIV infected individuals, who has lower cell mediated immunity, formation of granulomas cannot be seen as a defense mechanism. These conditions have increased neutrophyl activities and high bacteremia in turn. In HE stain, confusion between follicles and granulomas were eliminated by comparing the cellular density and cellular types in both. Follicles has germinal center along with dense lymphocyte areas in the periphery where as granuloma has thin area of epetheloid cells.

Among 40 cases of tuberculous lymphadenitis, all the cases showed presence of epetheloid cell granuloma along with the caseous necrosis; this was described as the diagnostic criteria of tuberculosis for tuberculous lymphadenitis by Paustian and Bockus. In those cases with granuloma, majority of the cases had 20-30% of the lymph node area occupied by the granulomatous area. And the maximum of 80% granulomatous area was observed in only 3 of the cases. In this study it was seen that the cases with higher percentage of granulomas had a very low percentage of caseous necrosis, where as the cases with higher percentage of necrosis had a lower percentage of granuloma.

Necrosis, typical of tuberculosis is the late manifestation induced by the phospholipid containing cell wall of Mycobacteria. Sayami *et al* has reported that in the presence of epetheloid cell cluster with caseation necrosis, the diagnostic accuracy is 100% while if there are epetheloid cells only the diagnosis falls to 80 % (TB lymph node). In this study, the samples showed presence of both granuloma along with caseous necrosis. However Das, Ghosh, *et al.* (1994) reports that the finding of epetheloid cell granuloma or the epetheloid cell clusters only cannot be considered as the diagnostic tool for tuberculosis, which can also be found in other granulomatous inflamations like sarcoidosis, fungal infections, cat scratch fever and parasitic infection.

Expression of T (CD3) cells and B (CD20) cells to a certain level by the lymph node is its function but for the identification of the pathological condition of lymph node requires costly reagents with highly skilled manpower.

Paracortical area and the interfollicular area consisted of T cells that were positive for CD3 stain. CD3 stain was found to be negative for the Langan's Giant cells. That means, the giant cells lack CD3 cells. Thus T cells were predominant in the granulomas that stained positive for CD3 cells was a result of cellular immunity. But only a small number of the cells in granulomas were found to be positive for CD20 marker.

As seen upon antigenic stimulation of Lymph nodes, it showed proliferation of T cells as CD3 stained positive cells in the diffuse cortex and B cells as CD20 stained positive cells in the follicles. The cortical area of the lymph node consisted of the Follicles in which different stages of B cells were found that were positive for CD20 stain. CD20 stain was found to have membrane positive character. Though follicles consist of B cells, secondary follicles with active germinal centers contain T lymphocytes positive for CD3 cells which was also observed in the specimens. Finding of T cells may be due to its migratory characteristic. S-100 proteins were not seen in follicular area but were found in the paracortex area that had a close association with T cells. In some of the specimen, S-100 activity was found higher where as in most of the specimens lower S-100 activity was observed.

According to Chakrabourty P (2001), the normal lymph node consists of 60-75% of T cells and 30-35% of B cells. Where as, according to Anderson (1990), the normal lymph node consists of 85% of T cells and 15% of B cells. The condition applied to the normal lymph nodes only but this study was conducted in infected or abnormal conditions. Due to presence of higher amount of necrosis in lymph nodes, it was difficult to interpret the CD3 and CD20 cells observed. Though among the cellular area, higher CD3 activity was observed than CD20 cells. Higher CD20 cells could also be observed in follicular hyperplasia following bacterial infections.

Diagnosis of tuberculosis lymphadenitis is dependent upon the impression of the individual pathologist. In Country like Nepal where tuberculosis is common and endemic due to limited

resources and frequency of the disease, diagnosis chiefly relies either on histology of biopsy or on cytology of FNA with or without in combination of AFB staining. presence of acid fast bacilli in acid fast stain showed the diagnostic accuracy of 100%.

## 6.2. Conclusion

In a country like Nepal where tuberculosis is highly epidemic, use of appropriate diagnostic tool for early, sensitive, specific and affordable diagnosis are essential.

Tuberculous lymphadenitis being common in country, combination of AFB stain with H-E stain could be useful diagnostic tools and techniques for the diagnosis of tuberculous lymphadenitis.

Immunohistochemical stain for the diagnosis of tuberculous lymphadenitis in combination with Haematoxylin and Eosin stain are useful, but requires more extensive analysis before generalization.

# 7. SUMMARY AND RECOMMENDATION

## 7.1 SUMMARY

This study was conducted by investigators of Central Department of Microbiology in collaboration with Patan Hospital during September 2002 to March 2003 with the objective to study the various biopsy features of lymph nodes from clinically suspected tuberculous lymphadenitis cases and to correlate them with Acid Fast stain, Haematoxylin stain and other Immunochemical stains.

1. Out of 40 clinically suspected tuberculous lymphadenitis cases, tuberculosis was confirmed with H-E stain.
2. The most susceptible age group was between 20-30 years of age.
3. Acid Fast Bacilli in ZN stained smears was detected in only 10% of the total cases.
4. Majority of the cases had a complain of fever (75%), followed by headache (27.5%), enlargement and tenderness (20%), loss of appetite (12%), shortness of breadth(5%) and the rest 7.5% had no complain at all.
5. Cervical lymph nodes were involved in 57.5%, auxiliary lymph nodes in 20%, submandibular in 10%, supraclavicular and linguinal in 5% each and extensive lymph node involvement in 2.5%.
6. Multiple lymph nodes involved were 80% and single node was involved in only 20% of the cases.
7. Giant cells were observed in only 95% of the cases.
8. Granuloma and caseous necrosis was present in all 40 suspected cases.
9. Lower percentage of granuloma was observed in lymph nodes with higher necrosis and higher percentage of granuloma was observed in lymph nodes with lower necrosis area.
10. Higher percentage of CD3 cells were present in paracortex, interfollicular area and granulomas where as lower percentage was present in fo;;icular area and it was absent in Lagerhan's giant cells.

11. CD20 cells were present in interfollicular area but it was absent in interfollicular area, giant cells and granulomas.
12. S-100 protein was found in only low percentage of cases in paracrtex area in close association with interdegitating reticulum cells, but it was absent in other lymph node areas.
13. combination of H-E and AFB staining was efficient in diagnosis of tuberculous lymphadenitis, although immunohistochemical staining were also sensitive.

## 7.2. RECOMMENDATIONS:

1. Immunohistochemistry should be combined with microscopy of Haematoxylin-Eosin stain of the lymph nodes.
2. Immunohistochemical staining alone may not be sufficient for the diagnosis of tuberculous lymphadenitis.
3. Immunohistochemical technique should be evaluated further with bigger number of samples before coming to final conclusion.

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# Appendix I

**COMPOSITION AND METHOD OF PREPARATION OF DIFFERENT STAINING REAGENTS USED FOR THE ISOLATION AND IDENTIFICATION OF ACID-FAST BACTERIA IN LYMPHNODE BIOPSY SAMPLES.**

**Staining Reagents:**

**1. Z-N stain for Mycobacterium bacillus (1882-1883) in tissue sections.**

| **Ingredients** | **gm/liter** |
|---|---|
| **Basic-fuschin** | |
| Carbol –fuschin | 1 gm |
| Phenol | 5 gm |
| Absolute alcohol | 10ml |
| Distilled water | 100ml. |
| **Acidified Methylene blue** | |
| Methylene blue | 0.25gm |
| Acitic acid | 1ml. |
| Distilled water | 100ml |
| **Acid alcohol** | |
| Hcl | 1ml |
| Alcohol (95%) | 100ml |

# Appendix II

## 2. Hematoxyline eosine staining

| Ingredients | gm/liter |
|---|---|
| Haematoxylin | 1g |
| Absolute alcohol | 10ml |
| Ammonium or potassium alum | 20 g |
| Distilled water | 200 ml |
| Mercuric oxide | 0.5g |

Dissolve the haematoxylin in the alcohol & add to the alum, previously dissolved in hot water. Bring quickly to the boil & add the mercuric oxide, when the solution will turn dark purple. Cool rapidly under the tap filter before use. The stain should be prepared in a flask of ample size on account of the frothing that takes place on addition of the mercuric oxide.

# Appendix III

**Age and sex wise distribution of Tuberculous Lymphadenitis.**

| Age Groups | Male | Male % | Female | Female % | Total |
|---|---|---|---|---|---|
| **0-10** | **2** | **5** | **1** | **2.5** | **3** |
| **0-20** | **2** | **5** | **5** | **12.5** | **7** |
| **20-30** | **4** | **10** | **9** | **22.5** | **13** |
| **30-40** | **3** | **7.5** | **3** | **7.5** | **6** |
| **40-50** | **1** | **2.5** | **5** | **12.5** | **6** |
| **>50** | **0** | **0** | **5** | **12.5** | **5** |
| Total | 12 | 30 | 28 | 70 | 40 |

# Appendix IV

**Result of AFB stain in tuberculous lymphadenitis cases.**

| AFB | Male | Female | Total No | % |
|---|---|---|---|---|
| Positive | **1** | **3** | **4** | **10** |
| Negative | **11** | **25** | **36** | **90** |
| Total | 12 | 28 | 40 | 100 |

## Appendix V

**Site and number of lymph nodes involved in Tuberculous lymphadenitis.**

| Site | Single | Multiple | Total | % |
|---|---|---|---|---|
| R Cer | **2** | **14** | **16** | **40** |
| L Cer | **2** | **4** | **6** | **15** |
| B/L Cer | **0** | **1** | **1** | **2.5** |
| Axillary | **2** | **6** | **8** | **20** |
| Submandi | **2** | **2** | **4** | **10** |
| Supraclavi | **0** | **2** | **2** | **5** |
| Linguinal B/L | **0** | **2** | **2** | **5** |
| Extensive all over | **0** | **1** | **1** | **2.5** |
| Total | 8 | 32 | 40 | 100 |

## Appendix VI

**Clinical findings of tuberculous lymphadenitis.**

| Clinical Symptoms | No. of Cases | % |
|---|---|---|
| **Fever** | 30 | 75 |
| **Weight Loss** | 10 | 25 |
| **Headache** | 11 | 27.5 |
| **Enlargement** | 8 | 20 |
| **Tenderness** | 8 | 20 |
| **Cough** | 5 | 12.5 |
| **Chest pain** | 5 | 12.5 |
| **Loss of Appetite** | 5 | 12.5 |
| **SOB** | 2 | 5 |
| **No Complian** | 3 | 7.5 |

## Appendix VII

**Statistical Tools:**

**Calculation of sensitivity, specificity, positive and negative predictive values.**

| Tests | True positive (TP) | True negative (TN) | False positive (FP) | False negative (FN) |
|---|---|---|---|---|
| AFB stain | 4 | 0 | 0 | 36 |
| Hematoxylin-eosine stain | 40 | 0 | 0 | 0 |

**CALCULATION OF SENSITIVITY**

Sensitivity was calculated as:

**Sensitivity**= TP/ (TP + FN) × 100%

**A. Sensitivity of AFB stain** = 4 / (4 + 36) × 100% = 10 %

**B. Sensitivity of Haematoxylin-Eosin stain** = 40 / (40 + 0) × 100% = 100%

**CALCULATION OF POSITIVE PREDICTIVE VALUE (PPV)**

PPV was calculated as:

**PPV**= TP / (TP + FP) × 100%

**A. PPV of AFB stain** = 4 / (4 + 0) × 100% = 100 %

**B. PPV of Hematoxylin-eosine stain** = 40 / (40 + 0) × 100% = 100 %

**CALCULATION OF PREDICTIVE VALUE OF NEGATIVE TEST (PPN)**

PPN was calculated as:

**PPN**= TN / (TN + FN) × 100 %

**A. PPN of AFB test** = 0%

**B. PPN of Haematoxylin-Eosin stain** = 0%

# Appendix VIII

## Questioniare.

**Clinical data**:

Name: Date:

Hospital number:

Age: Sex:

Sign and symptom:

Sample site:

Lymph node involved:

Diagnosis:

Reports on:

1. AFB:
2. Mountoux:
3. X-ray:
4. ELISA:

## Appendix IX

| Biopsy No. | Age | Sex | AFB | Granuloma % | Giant Cells | Necrosis % | CD3 % | CD20 | S100 | LN involved |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | |
| 90 | 20 | F | - | 30 | ≤5 | 8 | 75 | 30 | + | R Cervical (M) |
| 293 | 45 | F | - | 5 | ≤5 | 90 | 10 | 60 | - | Sub Mandibular (M) |
| 295 | 50 | F | - | 3 | ≤5 | 15 | 62.5 | 65 | + | Axillary (S) |
| 336 | 51 | F | - | 80 | ≥20 | 3 | 60 | 40 | + | R Cervical (M) |
| 401 | 1 | M | - | 60 | ≤5 | 17.5 | 30.5 | 65 | + | B/L Lingual (M) |
| 416 | 34 | M | - | 10 | ≤5 | 80 | 22.5 | 40 | + | R Cervical (S) |
| 1190 | 32 | M | - | 70 | ≤10 | 5 | 22.5 | 72.5 | + | Axillary (M) |
| 1713 | 18 | F | - | 60 | ≤5 | 15 | 50 | 25 | + | Axillary (M) |
| 1724 | 39 | F | - | 20 | ≤5 | 35 | 7.5 | 52.5 | + | R Cervical (M) |
| 1787 | 10 | M | - | 30 | ≤5 | 10 | 30 | 70 | + | L Cervical (S) |
| 2038 | 22 | F | - | 25 | ≤5 | 65.50 | 65 | 65 | + | Supra Clavicular (M) |
| 2153 | 50 | F | - | 20 | ≤10 | 15 | 62.5 | 10 | + | Axillary (M) |
| 2365 | 31 | F | - | 30 | ≥20 | 60 | 52.5 | 62.5 | + | R Cervical (M) |
| 2509 | 16 | M | + | 50 | ≥20 | 35 | 45 | 9 | + | R Cervical (M) |
| 2626 | 42 | F | + | 5 | ≥20 | 12 | 70 | 32.5 | - | Axillary (S) |
| 2745 | 21 | F | - | 60 | ≤10 | 30 | 75 | 75 | + | B/L Cervical (M) |
| 2914 | 28 | F | - | 50 | ≤15 | 30 | 55 | 68 | + | Sub Mandibular (M) |
| 2980 | 46 | F | - | -5 | ≤5 | 62.50 | 55 | 32.5 | + | L Cervical (M) |
| 3323 | 40 | M | - | 70 | ≥20 | 7 | 65 | 32.5 | + | Ext Multiple |
| 3590 | 16 | F | - | 60 | ≤15 | 25 | 55 | 45 | + | L Cervical (M) |
| 1731 | 20 | F | + | 25 | ≤5 | 20 | | | | R Cervical (M) |
| 548 | 33 | M | - | 5 | ≤10 | 90 | | | | Axillary (M) |
| 540 | 23 | M | - | 70 | ≤10 | 10 | | | | R Cervical (M) |
| 650 | 21 | M | - | 30 | ≤10 | 12.50 | | | | Sub Mandibular |
| 665 | 53 | F | - | 50 | ≤10 | 20 | | | | R Cervical ( S) |
| 691 | 19 | F | - | 20 | ≤5 | 62.50 | | | | R Cervical (M) |
| 711 | 46 | F | - | 30 | ≤20 | 30 | | | | L Cervical (M) |
| 794 | 21 | F | - | 80 | ≤15 | 20 | | | | Axillary (M) |
| 826 | 34 | F | - | 5 | ≤5 | 80 | | | | R Cervical (M) |
| 798 | 25 | F | - | 30 | 0 | 40 | | | | BL Lingual (M) |
| 936 | 26 | M | - | 35 | ≤5 | 20 | | | | R Cervical (M) |
| 1218 | 54 | F | ++++ | 20 | ≤5 | 60 | | | | R Cervical (M) |
| 3693 | 40 | F | - | 70 | ≤10 | 30 | | | | L Cervical (S) |
| 1181 | 23 | F | - | 20 | ≤5 | 65 | | | | R Cervical (M) |
| 2285 | 26 | M | - | 10 | ≤5 | 5 | | | | Axillary (M) |
| 1788 | 6 | F | - | 75 | ≤5 | 20 | | | | L Cervical (M) |
| 1293 | 22 | F | - | 5 | 0 | 90 | | | | Supra Clavicular (M) |
| 1566 | 18 | F | - | 50 | ≤15 | 5 | | | | R Cervical (M) |
| 1290 | 17 | F | - | 80 | ≤10 | 10 | | | | Sub Mandibular |
| 2138 | 5 | M | - | 40 | ≤15 | 55 | | | | R Cervical (M) |

R- Right.

L- Left.

M- Multiple Nodes.

S- Single Nodes.