CORRELATION OF TNF α EXPRESSION WITH MYCOBACTERIUM TUBERCULOSIS POSITIVITY AND DENSITY IN TUBECULOUS LYMPH NODE GRANULOMAS

Fiaz Ahmad, Abdurehaman kesrani, Ambereen Anwar

ABSTRACT

Background: Mycobacterium tuberculosis infection of humans is a disease of tremendous importance. Histopathology of the effected tissue is a time honored test for the diagnosis of disease. Due to vast number of granulomatous conditions demonstration of MTb in granuloma is important for definitive diagnosis.

Method: A crossectional study was performed at PGMI Lahore in Collaboration with department of pathologyAMC Abbottabad. Fifty diagnosed cases of tuberculous lymphadenitis were included in the study. Three sections of 3micron thickness were takenon different slides and stained for Auramin/Rhodamin stain, Zn Stain and one section was immunohistochemically stained for TNFα.Results were analyzed using SPSS 21 for Pearson's linear correlation.

Results: Statistical analysis show a strong negative correlation between TNFα expression and MTb positivity and density

Key Words: M. Tuberculosis, Auramin Rhodamin Stain

INTRODUCTION

Tuberculosis is an important disease of humans from time immemorial[1]. The disease has recently been discovered in Egyptian mummies[2]. These mummies appear to be 2100 years BC. In the modern world the story is even worse that the disease is responsible or 2 million deaths globally each year and 10 million cases each year[3]. Its importance has grown in the recent years that its co infection with other diseases such as AIDS has led to an increased mortality and morbidity[4]. Also important is the fact that drug resistance in MTb is causing more morbidity and mortality[5,6].

In Pakistan the situation is no different .according to WHO report 2013 Pakistan stands fifth in 21 contraries with high burden of tuberculosis. Estimated prevalence of disease is 350 cases per lack population. Incidence of tuberculosis was 258251 out of which 43416 were extrapulmonary cases. About 60,000 cases died of the disease in 2009[3].

The causative agent of tuberculosis is mycobacterium tuberculosis. Nine types have been discovered infecting humans in different regions of the world. These are further splitted into 36 subfamilies on the basis of pathogenicity and geography[7]. It was first discovered by Robert Koch in 1882 as causative agent of tuberculosis[8]. After a few years a reliable method of staining was devised by two scientists Franz Ziel and friedrich Neelson and the method was named after them as Ziehl-Nelson (ZN) staining method[8]. Unlike most common bacteria Mtb has different staining properties. This is due to its special cell wall with very high lipid contents[9]. Though it stains with difficulty but once stained it retains decolourization by weak acids and alcohols hence named as acid fast bacillus[10].

Staining of acid fast bacillus has been a challenge and many staining modifications were adopted to overcome this difficulty. Auramin / Rhodamin stain is one such stain showing better results over ZN staining method but detection of AFB in granuloma is still low[11,12].

In late 19th century Dr. William Coley described necrosis of tumors induced by bacterial toxins[13]. Later it was found to be an inducible factor in blood and tissues that is responsible for necrosis of tumors and has very important role in inflammation and was named Tumor necrosis factor[14]. Extensive study on this factor revealed that these are closely related group of pleotropic cytokines and TNFα is the prototype and most abundant of the group. It is secreted by many cell types such as macrophages, Lymphocytes, neutrophils, fibroblasts and even keratinocytes[15]. It has an important role in inflammation, immunity and expression of gene regulation.in pathological processes it has important role in cell proliferation, differentiation, apoptosis, modulation of gene expression and inflammation[16]. TNF α like many other cytokines has a pivotal role in defense against MTb regarding granuloma formation and activation of macrophages to launch a killing response against MTb. it has been seen in TNF α knockout mice that susceptibility to tuberculosis is increased and granuloma formation is poor with heavy loads of MTb, a disseminated disease and death.in humans it is observed that patients on TNFα therapy are susceptible to tuberculosis with poorly formed granulomas and rapidly disseminating disease[17]. It exists in both free and membrane bound

Pathology Department Ayub Medical College Abbottabad

Address for correspondence:
Dr. Fiaz Ahmad
Assistant Professor
Pathology Department Ayub Medical College Abbottabad
E mail. tanolilion@yahoo.com.

forms and both forms can lead to migration of lymphocytes and macrophages for granuloma formation[15]. In experimental gene knockout animal model deficient in TNF α exposure to MTb leads to rapid necrotic lesions with macrophages packed with MTb depicting an obvious failure to check the growth of and or killing of the bacteria[17].

Mycobacterium tuberculosis are killed in the body either by macrophage after ingestion or by other macrophages after apoptotic death of infected macrophage induced by TNFα. In case of deficiency of this cytokine infected macrophage undergoes a necrotic death conferring a growth advantage to bacteria in extracellular environment[18].

Among other factors responsible for low detection of MTb in granuloma TNFα may be one of them as high expression of this cytokine causes bacterial death by macrophage itself or by apoptosis of heavily infected macrophages and killing by surrounding ones[18]. Conversely its low expression may lead to ample multiplication and hence easy detection of MTb in granuloma.

MATERIALS AND METHODS

A crossectional study was conducted at PGMI Lahore between 27th December 2011 to 18th April 2014 in collaboration with department of pathology Ayub medical college Abbottabad. A total of 50 diagnosed cases of tuberculous lymphadenopathy with no other pathology were included in the study. Cases were taken by a nonprobability sampling method.

Four slides were cut from each block at 3μ thickness. One slide was routinely stained for H & E for confirmation of granulomas 2nd was stained for AFB with ZN staining Technique(19) and 3rd was stained for AFB with Auramin/ Rhodamin stain(19). Fourth slide was stained for expression of TNFα with annti human TNFα rat antibody targeted by anti- antihumananTNFα antibody produced in Rabbit. The 2ndry anti body was fluorescent. Immunostain was purchased by Abcam® through a local vender. Product code for primary antibody was ab6671 and ab6717 for 2ndry antibody. Immunostain was standardized on sections of inflamed appendix. From the primary stain 200μl each of 1/100 and 1/200 dilutions were prepared and from 2ndry 1/300 and 1/400 each dilutions were prepared. Dilution medium was phosphate sodium chloride/ sodium azide buffer at p^H 7.2 supplied with the kit.

Four slides of acutely inflamed appendicular tissue were taken and quenched with 1/50 diluted calf serum for 45 minutes. Different combinations of primary and secondary staines were used on each slide following the staining instructions provided by the vender. The best results were seen with 1/200 primary and 1/400 secondary antibody. Presence of greenish yellow fluorescence was taken positive for TNFα.

Quantification of TNFα was done by taking 10 random pictures at 10X magnification from each slide. The pictures were then opened in Adobe Photoshop 7 software and yellowish green pictures were than calculated and expressed as percentage of the total. Total picture size was 4 megapixels. TNFα expression was expressed as average percentage of 10 readings.

RESULTS

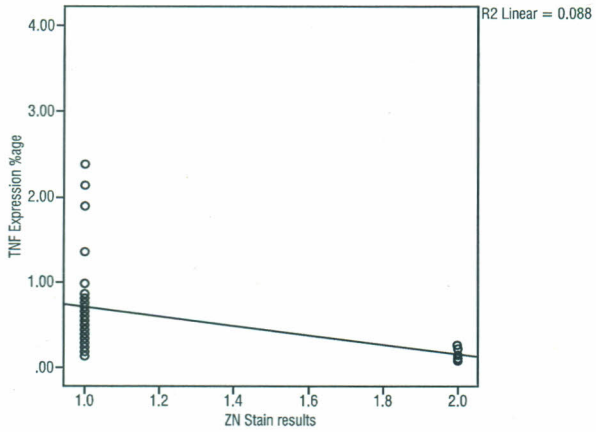
The study comprised of 50 diagnosed cases of tuberculous lymphadenitis. The patients had a mean age range of 9-80 years. Sex distribution was 33:17 female and male respectively. Acid fast bacillus was positive in 18% and 42% respectively with ZN and Auramin / Rhodamin stains. Auramin/Rhodamin stain was more sensitive and had picked all the cases positive on ZN stain and had picked an additional 24% cases.

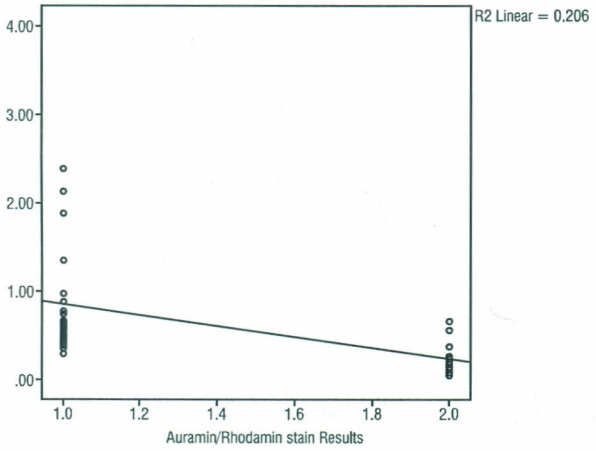
Out of the 18% Cases positive on ZN stain 12% had low load of bacteria of 1-5/OIF in an average of 10 fields. Two pecent had an average load of 6-10/OIF, another 2% had a load of 11-15/OIF and 2% showing a load of more than 15/OIF. In cases positive with Auramin/ Rhodamin stain 22% had alow load of 1-5/OIF,10% a load of 6-10/OIF, 6% showing load of 11-15/OIF and 4% a load of more than 15/OIF.

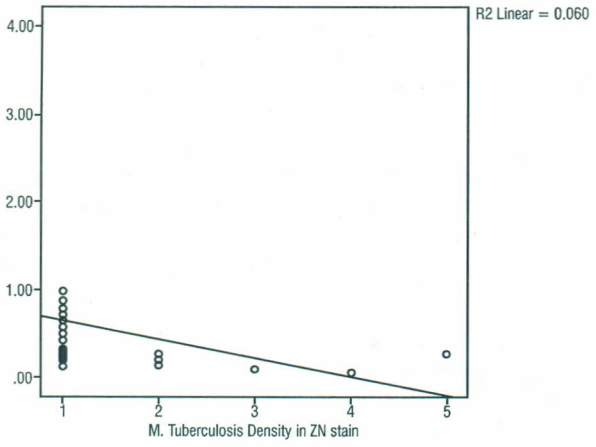
Mean TNFα expression was 0.60% ± 0.68 with arrange of 0.07—3.95%. Pearson correlation was used for TNFα expression and bacterial positivity/load. A negative correlation was observed between TNFα expression and bacterial positivity in ZN staining Method. The p value was less than .05 hence statistically significant. Similarly a strong negative correlation between TNFα expression and bacterial positivity in Auramin/ Rhodamin stain was observed. The p value was less than .05 hence statistically significant. Pearson's correlation between TNFα expression and bacterial load in Zn staining method shows a negative correlation but with a p value of greater than .05 hence statistically not significant wear as we observed a strong negative correlation between TNFα expression and bacterial density in Auramin/Rhodamin stain and p value was less than .05 hence statistically significant.

DISCUSSION

Among Bacterial infectious diseases tuberculosis is very important because of its rapidly spreading nature and morbidity and mortality it causes as a single infection as well as a confection[20]. Being a treatable disease early diagnosis holds a key to treatment and avoiding complications[21]. Histopathology is important diagnostic test especially in extrapulmonary location. Demonstration of AFB is very important regarding definitive diagnosis because of large differentials of granulomatous diseases[22]. Staining yield of AFB is usually low in granulomas. Many factors may be responsible for this low detection and TNFα may be one of them. Tumor necrosis factor alpha is a cytokine that modulated inflammatory and immune response of the

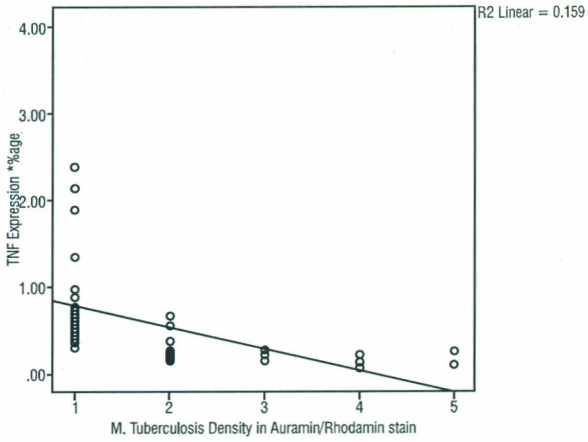






individual and has diverse functions. It has a pivotal role in defense against mycobacterial infection[23].

Results show that most population effected was below 40 years(86%). And is consistent with the findings of Munynch etal 2009[24] and update 2013. In our study disease was more prevalent in females (66%) results are not in agreement with Munynch et al.[25] who claims a higher prevalence in males, however a regional study conducted in India by Mukherjee shows a higher prevalence(54%) in females.



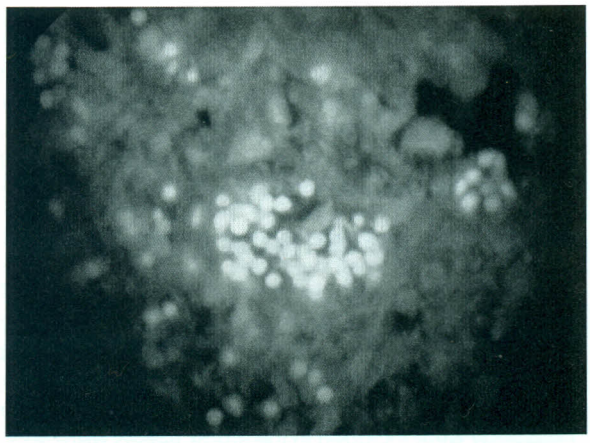


Figure 1: TNF α expression in lymphocytes in a granuloma

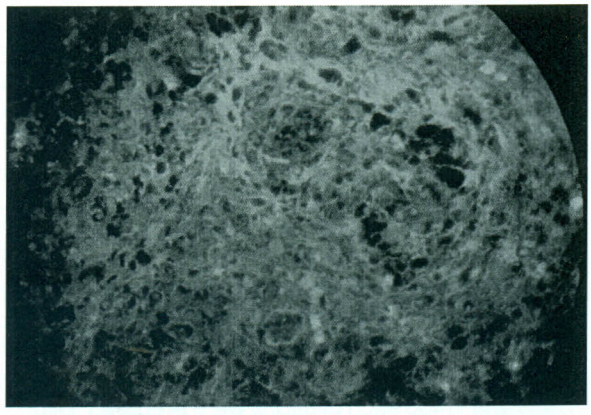


Figure 2: low TNF α expression i a granuloma

Lower positivity of ZN stain is in accordance with most national and international studies[26]. Auramin /Rhodamin stain was positive in 42% of the cases. many studies like Ulukanligil et al.[27] and Shrestha. et al.[28] shows a positivity of 82% and 71% respectively in smear and Ghenaath et al.[29] show 26% positivity . study conducted by shrestha et al was done on cases proven positive on mycobacterial culture. His gross result including culture negative cases was 36% close to our

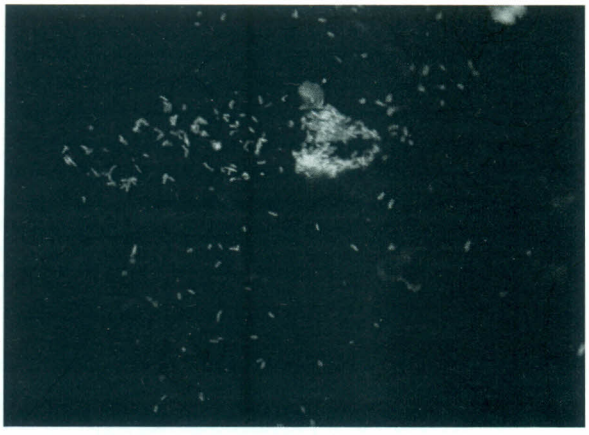


Figure 3: M tuberculosis Auramin/Rhodamin stained section

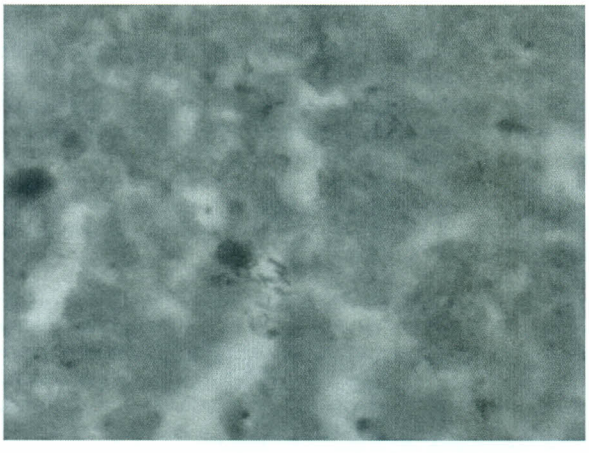


Figure 4: M tuberculosis ZN stained section

study. The study of Ghennath etal may be low because of smaller sample size he slected for his study.

TNFα quantification was done by photographing the immunostained slides under UV light, processed in Adobe Photoshop 7 to get percentage expression. Digital imaging and image processing in Adobe photoshop are newly discovered tools for anatomical pathology. Quantification of target antigen in immunohistochemistry with the help of Adobe photoshop is in practice since late ninties as Lehr et al.[30] developed a similar technique to quantify ER & PR reseptars in breast cancer. More work was also done by Long etal in2004[31].

A strong negative correlation was seen between Mycobacterial density / positivity and TNFα expression in our study. Many international studies Bourigault et al[32]: Kapoor et al.[33] have proved that optimal expression of this cytokine restricts growth and multiplication of Mycobacterium tuberculosis in granulomas. Similarly Mohan et al.[34], Olleroset et al.[35]: Lin et al.[36] have depicted that inhibition of this cytokine leads to multiplication and dissemination of mycobacteria in tissue. Hence the results of our study are in accordance with most studies conducted in this regard.

CONCLUSION

Results of this study show that there is a negative correlation between Mycobacterium Tuberculosis positivity/density and Tumor Necrosis factor alpha expression in tuberculous granulomas. This may be one of the many causes for low detection of M Tuberculosis in granulomas. Further studies are needed to evaluate all the factors responsible for low detection in wax embedded tissues.

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