ANTI-BCG Immunohistochemical Detection of Mycobacteria in Formalin-Fixed Paraffin-Embedded Tissue Samples if Granulomatous Lymphadenitis

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Abstract

Objective: In order to improve the diagnosis of extrapulmonary tuberculosis (EPTB). The present study has designed to evaluate the applicability of immunohistochemistry (IHC) by using polyclonal anti-BCG (Bacillus Calmette Guérin) antibody on tissue sections for detection of tuberculous bacilli or their components in granulomatous lymphadenitis and to assess its advantages over conventional Ziehl Neelsen (ZN) histochemical staining.

Materials and Methods: A retrospective study of forty one formalin-fixed and paraffin-embedded (FFPE) biopsies of tuberculous granulomatous lymphadenitis, randomly selected from the archives of pathology laboratories in Erbil during the period from 2009 -2012. All specimens were subjected to ZN staining for acid fast bacilli (AFB) and IHC staining by polyclonal anti-BCG antibody; then a comparison of the detection rate of both techniques was done.

Results: AFB were identified by ZN stain in 27/41 (65.9%) of the cases; while IHC staining was positive in 39/41 (95.1 %) of cases.

Conclusion: IHC is a simple, sensitive and useful diagnostic technique, superior to conventional ZN staining, in detection of mycobacterium bacilli or their components in granulomatous lymphadenitis.

Keywords: Tuberculous granulomatous lymphadenitis, Immunohistochemistry, Anti-BCG antibody.
Running title: Anti-BCG IHC of Granulomatous lymphadenitis.

Introduction

Tuberculous lymphadenitis is the most common form of EPTB [(1)], accounting for approximately 10–15% of all tuberculosis infections and occurs in up to 50% of patients with human immunodeficiency virus (HIV)-tuberculosis co-infection. The annual incidence rates of EPTB have increased not only in developing countries but globally over the last few years [(1-4)]. *Mycobacterium tuberculosis* and, to a lesser degree, *Mycobacterium bovis* have previously been supposed to be the most common causative agents of tuberculous lymphadenitis [(5)]. However, a substantial number of cases of granulomatous lymphadenitis are caused by non-tuberculous mycobacteria, especially in countries with high prevalence of these mycobacteria [(6, 7)].

The diagnosis of EPTB has always been a problematical and histological examination is usually required for the diagnosis as the clinical criteria used for diagnosis have poor sensitivity

and specificity and may lead to over-diagnosis, especially in countries with high endemic rates of tuberculosis [8]. The histological diagnosis usually depends on the detection of the classical histological changes of granulomatous inflammation suggestive of tuberculosis. These histological features can be found in various conditions and diseases other than tuberculosis. Therefore; the definitive diagnosis of tuberculosis is dependent on the demonstration of AFB by ZN staining. The yield of this method is limited as most EPTB is paucibacillary [6, 9 and 10] and fresh unfixed tissue with live bacilli is usually not available for culture. Moreover, culture takes several weeks and is often negative in EPTB. Thus, there are samples that are negative for both acid fast staining and culture. Moreover; in immune-compromised tuberculous patients, the histological features can be atypical, leading to considerable difficulty and delay in diagnosis [3, 11] and an incorrect diagnosis of tuberculosis leads to increased morbidity and mortality due to suboptimal or wrong treatment that has significant economic implications. Also, because effective as well as specific chemotherapy is available for this potentially curable infectious disease, clinicians cannot delay antituberculosis treatment while waiting for a confirmatory bacteriological diagnosis of AFB. Therefore; there is a great need to develop a better diagnostic test to provide an alternative to AFB microscopy and culture for better clinical management.

Detection of mycobacterial antigens by IHC, using polyclonal and monoclonal antibodies raised against whole organisms or its purified components in cell wall or cytoplasm of mycobacteria, is an alternative to conventional acid-fast staining with varying results on paraffin embedded tissues [9, 12-15]. The polyclonal rabbit anti- BCG is a commercially available antibody raised against the Bacillus Calmette- Guérin, an attenuated strain used to immunize against *Mycobacterium tuberculosis* infections, containing a substantial number of shared antigens with other mycobacteria species and is considered superior to histochemical stains and culture in detection of mycobacteria [14].

In the present study, we investigated the diagnostic potential of IHC on tissue sections for specific detection of mycobacterial antigens in the cytoplasm of monocytoid epitheloid cells (macrophages) in lymph nodes with histological features of tuberculous granulomatous inflammation.

Materials and Methods

A retrospective study of forty one FFPE lymph node biopsies, that were obtained by surgical resection and contained histologically caseous and non caseous granulomatous inflammation , were randomly selected from the archive's tissue block collection of different public and private pathologic laboratories during the period from 2009 -2012 , after approval by Institutional ethical committee. Majority of lymph nodes examined were from the cervical region. Diagnosis of tuberculous lymphadenitis was based on thorough clinical examination, routine hematological and serological investigations, chest X-ray, and lymph node biopsy that followed by histopathological examination. Conclusion of tuberculous granulomatous lymphadenitis was based on the presence of caseous and non caseous granulomatous adenitis in tissue sections. Presence of AFB in the tissue section was not considered as a gold standard to

confirm the diagnosis and mycobacterial culture of the biopsied tissue was not done due to lack of facilities. Specimens containing the largest granulomatous lesion in each case were selected; and depending on granulomatous changes observed on histopathological examination of Hematoxylin and Eosin (H and E) staining, a comparison was made between ZN stain and IHC stain regarding the detection of mycobacterium; both were analyzed under high power lens (X 400) of light microscope (Olympus, Tokyo, Japan).

Immunohistochemistry

Immunostaining has carried out by the standard Avidin-Biotin Complex peroxidase-antiperoxidase method and by using the EnVision+System-HRP (Dako Cytomation, Denmark) as the manufacturer's instructions described in the leaflet supplied with the antibody. Briefly, after deparaffinization and rehydration, the sections were microwaved in 10mM citrate buffer (pH 6.2) for 15 min at 95 °C. The sections were cooled for 20 min at room temperature and then were treated with 3% hydrogen peroxide for 10 min in order to block the endogenous peroxidase activity. Primary antibody— rabbit anti-BCG polyclonal antibody (Dako, Glostrup, Denmark) code no. B0124 Lot 115 in 1:500 dilution, were then applied to the sections and incubated overnight at room temperature. This step was followed by washing and a 40-min incubation with anti-rabbit dextran polymer conjugated with streptavidinbiotin peroxidase complex (LSAB+ system; Dako Cytomation®, Denmark) at room temperature. Visualization was performed as a brown reaction with diaminobenzidine (DAB) containing H2O2 as a substrate, applied for 10 min. Sections were counter-stained with Mayer's haematoxylin, dehydrated, cleared in xylene, mounted with DPX mounting and visualized under a light microscope (Olympus, Tokyo, Japan). Pulmonary tuberculosis with a high bacillary index in ZN stain was used as a positive control while two negative controls were used, first; a specimen of tuberculous lymphadenitis where the primary antibody was omitted and the second specimen with a diagnosis of foreign body granuloma. Brown color reaction products indicate positive results.

This antibody reacts with about 100 different BCG antigens of which many are common to other mycobacteria. A few of the antigens are even common to bacterial species of families different from Mycobacterium[14].

Results

The lymph node biopsies were for 22 male and 19 female patients. Their ages ranged between 10 and 65 years and the mean was 35years.

Histology and ZN staining for AFB

Both caseous and non-caseous granulomas with epithelioid cells, lymphocytes and multinucleated giant cells characteristic of tuberculosis were observed in all 41 cases with the conclusion of tuberculous granulomatous lymphadenitis was made on H and E staining. Necrotic caseous granulomas were found in 30/41(73.2%) of the patients, whereas 11/41 (26.8 %) showed predominantly non-caseous granulomas. By the ZN staining method, AFB were demonstrated in

27 (65.9%) of lymph node specimens, while 14(34.1%) of specimens were negative (table 1), AFB were solid, beaded present in the granuloma in association with epitheloid cells as well as in the caseous areas. No culture results were available for all cases.

Table 1: Results of ZN histochemical staining and Immunohistochemistry

		ZN staining		Total
		+ve	-ve	
IHC	+ve	27	12	39
	-ve	0	2	2
Total		27	14	41

Immunohistochemistry

By immunostaining with Dako anti-BCG antibodies, 39/41 (95.1%) cases of granulomatous lymphadenitis showed positive immunostaining for mycobacterial antigens (table 1) in the form of brownish granules in and around granulomas and in the cytoplasm of macrophages and giant cells. About 75 to 80% of epitheloid cells in the sections showed positive cytoplasmic immunostaining or mycobacterial antigens. Besides this, aggregates of immunostained extracellular brownish material was also seen contrasted sharply with the rest of the amorphous necrotic debris. None of the negative controls showed positive immunostaining, indicating that nonspecific immunostaining did not occur by this technique. Only two (4.8%) cases were negative for both ZN andanti-BCG immunostaining.

Discussion

Improved methods to detect and manage EPTB, such as new diagnostics, drugs, and vaccines, are required to achieve the goal of the World Health Organization to eradicate tuberculosis [16]. The diagnosis of EPTB is often difficult and, apart from clinician evaluation, it depends on several different techniques like bright light microscopy based on conventional H and E stain, and the demonstration of the causative agent of the disease, i.e. *Mycobacterium tuberculosis*, in tissue specimens by different techniques such as the ZN stain, mycobacterial culture, IHC and polymerase chain reaction (PCR) [8,10]. Each of these techniques has advantages and limitations. For example Hand E stain shows granulomatous inflammation which is a distinctive pattern of chronic inflammation that encountered in a number of immunologically-mediated, infectious and non infectious conditions [17]. Mycobacterium tuberculosis is the leading cause of infectious granulomtous disease especially if the granuloma shows caseous necrosis and in endemic areas, any granulomatous inflammation is considered TB till prove otherwisew; however other etiologies can be implicated as Cat-scratch disease, leprosy, syphilis, some mycotic infections ---etc. [17,18]. All the lymph node biopsies involved in this study had shown caseous and non caseous granulomatous inflammation with the conclusion of tuberculous granulomatous lymphadenitis was made on Hand E sections.

Histochemical ZN stain is a rapid technique usually used for detection of mycobacterial infection in tissue sections with granulomatous inflammation; but frequently presents negative results due to the fact that only intact bacilli can take the stain and it can be positive when there are more 10,000 organisms per ml of sputum [9, 10]. In this study ZN stain was positive in 27/41 (65.9%) of cases .This is higher than others studies [19, 20], mostly because ZN stain had been done before initiation of anti-mycobacterial therapy which is known to change the capsule's integrity and prevents acid fast staining, while in 14 cases with granulomas, the ZN stain was negative mostly because the bacilli was of small quantity or partially treated.

IHC is a simple procedure that has been used to identify mycobacteria in sputum and tissue sections from lung, brain and lymph nodes with higher sensitivity than ZN staining as the presence of an intact bacillary cell wall is not a prerequisite [9,13,15,19,20].

In this study, we used a commercially available anti- BCG antiserum (Dako) as the primary antibody that showed positive immunoexpression in 39/41(95.1%) which is higher than the detection rate by ZN stain indicating that the concentrated debris derived from mycobateria apparently retained its antigenic property although it had lost its AFB staining property. This result was similar to many other studies [19, 20], reflecting the possibility of detection of fragmented bacilli in IHC [9, 15, 21]. Only two (4.8%) cases were negative for both ZN and anti-BCG immunostaining, indicating that the diagnosis of tuberculous lymphadenitis need to be revised or confirmed by other more specific techniques. The drawback of polyclonal anti-BCG immunostaining is inability to differentiate between different species of mycobacterium which is important as the incidence of mycobacteria other than tuberculosis in immune-compromised AIDS patients is increasing [6, 7]. In addition to its cross reactivity with other bacterial and fungal antigens [14]; however the sensitivity of IHC test in this study was based on both the immunologic identity of the mycobacterial antigens and the distribution of those antigens within the classical granulomatous inflammation that were useful in establishing the mycobacterial etiology of caseating granulomas in lymph nodes especially in cases with a low bacterial load or partially treated.

Other techniques that can be used for detection of *Mycobacterium tuberculosis*, as culture and PCR [22], are costly and not available for routine use in our locality. Recently, the rapid diagnosis of tuberculosis by nucleic acid amplification using the PCR technique has become feasible in fresh material, and has also been used for formalin-fixed paraffin-embedded tissue [22,23]; but they are not relevant because they are complicated, expensive, requiring sophisticated equipment, time consuming and are not freely available for routine use in our locality.

Conclusions

The results indicate that anti-BCG immunostaining, in comparison to ZN histochemical stain, is a suitable technique for the rapid identification of mycobacterial antigens in paraffin-embedded specimens and in establishing the mycobacterial etiology of caseating granulomas. Also it is easily performed and suitable to laboratories in developing countries where laboratory resources are limited.

Disclosure: There is no conflict of interests and the work was not supported or funded by any drug company.

CONFLICT OF INTERESTS.
There are non-conflicts of interest.

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الخلاصة

الهدف: لاجل تحسين تشخيص التدرن خارج الرئوي , تم اجراء هذا البحث لمعرفة دور الكيمياء النسيجيه المناعيه وذلك باستعمال مضادات متعدده ضد (BCG) المطبقة على المقاطع النسيجية لغرض معرفة وجود عصيات التدرن الرئوي او مكوناتها في العقد اللمفاوية ومعرفة فوائدها مقارنة بالصبغة الكيميائية (Ziehl Neelsen).

المواد والطرق: دراسية تراجعية لواحد واربعين مقطع نسيجي مثبتة بالفورمالين ومغطسة بالبارافين لالتهاب العقد اللمفاوية بالسل ، والتي اختيرت عشوائيا" من ارشيف مختبرات الباثولوجي وخلال الفترة من ٢٠٠٩ الى ٢٠١٢. كل النماذج نم صبغها بالصبغة الكيمياثية (Ziehl Neelsen) والصبغة الكيميائية النسيجية المناعية باستعمال مضادات متعدده ضد (BCG) ومن ثم مقارنة النتائج النهائية لكلا التقنيتين.

النتائج:- لقد وجدت عصيات التدرن في (٤١/٢٧) (٦٥,٩%) من الحالات باستخدام بالصبغة الكيمياثية (Ziehl Neelsen) بينما وجدت في (٤١/٣٩) (٩٥,١%) من الحالات باستخدام تقنية الصبغة الكيميائية النسيجية المناعية .

الأستنتاجات :- تقنية الصبغة الكيميائية النسيجية المناعية بسيطة و حساسة و مفيدة وافضل من الصبغة الكيمياثية (Ziehl Neelsen في تشخيص عصيات التدرن أو أجزائها في العقد اللمفاوية .

الكلمات الدالة: عقد لمفاوية متدرنة، الصبغة الكيميائية النسيجية المناعية، أنتي ال BCG أجسام مضادة .