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IMMUNOHISTOCHEMICAL STUDY OF INDUCIBLE NITRIC OXIDE SYNTHASE "iNOS" IN PERITONEAL TUBERCULOUS GRANULOMA

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ABSTRACT

Background/Aim: Nitric Oxide (NO) is important in host defense against *Mycobacterium tuberculosis* in rodents, but the presence of high-output NO production in human tuberculosis has been controversial. This study aimed to investigate iNOS expression by peritoneal macrophages in TB peritonitis and to gain insights into the structural properties of peritoneal TB granuloma**.**

Patients and Methods: Peritoneal biopsies were obtained from 28 undiagnosed cases of ascites and examined histopathologically by H&E stain. Accordingly, specimens proved to be TB peritonitis were then immunohistochemically stained for iNOS, the macrophage marker CD68 and CD3 and CD20 as markers of T and B lymphocytes respectively. Eight control cases of normal peritoneum were included.

Results: TB peritonitis was diagnosed in 16 cases. TB granulomas were found in 9/16 cases (56%) and a diffuse granulomatous reaction was found in the remaining7/16 cases (44%). Immunoreactivity to iNOS and CD68 were intensely expressed in macrophage rich TB granuloma and in the diffuse granulomatous TB reaction. Most Langhans cells (multinucleated giant cells) showed strong reactivity to both CD68 and iNOS. In TB granuloma, $CD3^+$ cells were found at the periphery with few $CD20^+$ cells in its center. Control cases showed complete negativity for iNOS, CD3, very small number of CD68 and/or CD20 cells.

Conclusion: In TB peritonitis, an increased local expression of iNOS in granuloma associated macrophages of untreated patients indicating excess NO production in the active stage of this form of Tuberculosis. Further studies are needed to test the therapeutic implications of NO in different forms of TB.

Keywords: *Peritoneal TB granuloma, immunohistochemistry, iNOS, CD68, CD3.*

INTRODUCTION

Even in the 21st century, tuberculosis (TB) causes illness or death of more than eight or two million individuals, respectively, every year, with the highest incidences of morbidity and mortality in Sub-Saharan Africa[1] and South East Asia [(2)]. In Egypt, every year the National Tuberculosis Control Programme (NTP) of the Ministry of Health and Population (MOHP) register more than 12,000 new TB patients [(3)].

Tuberculosis can involve any part of the gastrointestinal tract from mouth to anus, the peritoneum and the pancreaticobiliary system. It can have a varied presentation, frequently mimicking other common and rare diseases [(4)]. Peritoneal involvement may occur from spread from lymph nodes, intestinal lesions or from tuberculous salpingitis in women. Abdominal lymph nodal and peritoneal tuberculosis may occur without gastrointestinal involvement in about one third of cases [(5)]. Peritoneal tuberculosis remains a significant problem in parts of the world where tuberculosis is prevalent. In a series of two hundred Egyptian patients with undiagnosed ascites, tuberculous peritonitis was diagnosed in 45% of them [(6)].

Increasing population migration, usage of more potent immunosuppressant therapy and the acquired immunodeficiency syndrome epidemic has contributed to a resurgence of this disease in regions where it had previously been largely controlled. Tuberculous peritonitis frequently complicates patients with underlying end-stage renal or liver disease that further adds to the diagnostic difficulty[(7)]. The disease is unusual in patients without risk factors. In these conditions the diagnosis of tuberculous peritonitis is often delayed, resulting in high morbidity and mortality [(8)].

Mycobacterium tuberculosis is an intracellular pathogen that readily survives and replicates in human macrophages[(9)]. It produces latent infection or progressive disease. Indeed, latent infection is more common since it occurs in one-third of the world's population. TB is the unique disease in that 90% of all infections remain latent, 5-10 % of infected individuals develop clinical disease and 5% of those that had suppressed it initially reactivate infection during their lifetime[(10)].

The host effector mechanisms against *M. tuberculosis* infection are not well understood, and this remains a problem in the development of new vaccines and immunotherapies in tuberculosis[(11)]. At present, the main candidates for the direct killing of *M. tuberculosis* are granulysin produced by T-cells and nitric oxide and the superoxide radical produced by activated macrophages[(12, 13)]

Previous studies investigated the human immune response against *M. tuberculosis* infection mainly *in vitro* using peripheral blood mononuclear cells (PBMCs) from TB patients and healthy purified protein derivative positive (PPD+) control individuals [14]. However, since the original investigations by classical pathologists around the turn of the 19th century, only scattered reports have focused on the local pulmonary immune response in human tuberculosis [15, 16, 17].

Most studies aimed at dissecting the local immune response have been performed in animal models, notably in the mouse [18], guina pig [19], and more recently investigating chemokines and cytokines in non-human primates [20]. However, conclusions from animal models to patients need to be drawn with great care and the local cross-talk between *M. tuberculosis* and the immune system, which is responsible for the development and maintenance of granulomatous lesions, has not been satisfactorily examined in tuberculous patients. Moreover, all published reports described the local immune response in the lung tissue [21] or lymph nodes[22] and to the best of our knowledge; no published work has described the local immune response in peritoneal tuberculosis.

The role of nitric oxide (NO) in the host-defense against human tuberculosis (TB) is controversial. Although experimental evidence indicates that NO may play an important role in controlling TB, its expression in human tuberculosis has not been systematically characterized[23]. However, there is a growing body of evidence that NO produced by TB-infected macrophages and by epithelial cells has antimycobacterial effects against *M. tuberculosis*. The precise mechanism(s) by which NO and other reactive nitrogen species antagonize *M. tuberculosis* is not known, but may involve disruption of bacterial DNA, proteins, signaling, and/or induction of apoptosis of macrophages that harbor mycobacteria. It also appears that certain strains of *M. tuberculosis* have evolved strategies to combat the toxic effects of NO [24].

The common model of a human tuberculous granuloma describes an area of central necrosis, which provides the nutritional source for persisting mycobacteria, surrounded by a dense leucocytes wall preventing mycobacterial spread. Leucocyte infiltration also contributes to massive impairment of the affected tissue [21]. A better understanding of the host immune response against *M. tuberculosis* is crucial to elucidate the alternative disease outcomes [10].

In the current study we tried to 1- Determine whether inducible nitric oxide synthase "iNOS" expression by peritoneal macrophages is increased in patients infected with peritoneal TB. 2- Characterize the cell types in TB granulomas by studying the expression of CD68, CD3 and CD20 immunostaining as markers for macrophages, pan T and pan B lymphocytes respectively to gain insights into the structural properties of peritoneal TB granuloma.

PATIENTS & METHODS

The current study included 28 patients with undiagnosed ascites referred to the Departments of Tropical Medicine and Gastroenterology, Assiut and Sohag University Hospitals. For all patients complete blood count, ESR, C-reactive protein, chest X ray were performed. Ascitic fluid samples were collected for detection of cells and determination of proteins and serum-ascites albumin gradient (SAAG) was calculated for each of them. An abdominal ultrasound examination was performed.

Laparoscopy (Pentax EPM 3300 with videomonitor WV-CM 2000) was done and peritoneal biopsies for histopathologic examination were obtained from all patients. Suggestive signs of tuberculosis at laparoscopy as described by Sharma and Bhatia[25] include:
a) Thickened peritoneum with tubercles.
b) Thickened peritoneum without tubercles.
c) Fibroadhesive peritonitis with markedly thickened peritoneum and multiple thick adhesions fixing the viscera

Biopsy specimens obtained from all patients were formalin fixed and paraffin embedded. Five-micron tissue sections were made for hematoxylin and eosin (H&E) and Ziehl-Neelsen (ZN) staining (when needed) according to a standard protocol [26] to confirm their final diagnosis. An immunohistochemical study was then performed for the resulting tuberculous specimens and for 8 control cases of peritoneum removed with surgically excised organs sent to our histopathology laboratory (e.g. with excised tumors).

Immunohistochemical staining for iNOS, CD68, CD3 and CD20:

Sections were deparaffinized, rehydrated, and washed in phosphate buffered saline (PBS, pH 7.2). The sections were heated in a microwave oven in citrate buffer (pH 6.0) for 15min. Endogenous peroxidase was blocked by 5% hydrogen peroxide for 5 min, followed by washing for 5 min in PBS. The sections were pre incubated in 1% bovine serum albumin (BSA) for 30min at 37 C° and then incubated overnight with the primary

antibody for iNOS in a dilution of 1/50, and CD68 in a dilution of 1/100, and CD3 in a dilution of 1/100 and CD20 in a dilution of 1/200. They are rabbit polyclonal antibody raised against human species; (Catalogue # RB 9242-P0, LabVision Corporation, USA) for iNOS & mouse monoclonal antibodies raised against human species (Catalogue # MS 397-P0, # MS 401-S0, # MS 340-S0, LabVision Corporation, USA) for CD68, CD3 and CD20 respectively, diluted in PBS with 1% BSA. The slides were then washed with PBS, and incubated for 60 min with a biotinylated secondary antibody (Catalogue # TP-015-HD, LabVision Corporation, Westinghouse, USA). The sections were washed again in PBS, and incubated with 14-diaminobenzidine and 0.06% H_2O_2 for 5 min. They were counterstained with hematoxylin, dehydrated in alcohol, cleared in xylene and cover slipped.

According to the manufacturer instructions, sections of lungs, tonsils (available in our laboratory) were used as the positive control for iNOS and CD68 respectively, whereas specimens of lymph nodes with reactive lymphoid hyperplasia were used as the positive control for CD3 and CD20. Negative controls were performed by omitting the primary antibody each time.

Reactivity would be either cytoplasmic (iNOS and CD68) or membranous (CD3 and CD20). Cells with cytoplasmic brownish iNOS immunoreactivity or light brown granular CD68 positivity, light brown membranous CD3 or CD20 staining were considered positive. Immunostaining was evaluated by bright field light microscope; low power magnification (X40, X100) to specify the lesion and high power magnification (X200, X400) to evaluate the immunostaining. When more than 50% of granulomatous tissue expressed iNOS or CD68, the case was considered positive and the expression was carefully evaluated as focal or diffuse, and the intensity of staining was evaluated as weak, moderate or strong.

RESULTS

According to clinical, laparoscopic (Fig. 1) and histopathologic results, patients were: 16 TB peritonitis, 11 malignant ascites and one case with peritoneal bilharzial granuloma (Fig. 7). These results are illustrated in Table (1).

TB peritonitis patients were 14 females and 2 males. Their ages ranged from 17 to 60 years, with a mean of 35±20 ys. Their presenting symptoms and signs are summarized in Table (2). Abdominal ultrasonographic findings at presentation were either the presence of ascites with adhesions in 10 (62.5%), turbid ascites in 2 (12.5%) and turbid ascites with adherent intestinal loops in 4 (25%) patients. Pleural effusion was detected in 2 (12.5%) patients and adenexal mass in 1(6.3%) patient.

HISTOPATHOLOGICAL RESULTS

Among sixteen patients proved to have TB peritonitis by H&E and Ziehl-Neelsen staining, granulomas (Fig.2) were found in 9/16 (56.3%) and composed of well-defined collar of lymphocytes surrounding epithelioid cells (macrophages), lymphocytes and Langhans giant cells with eosinophilic cytoplasm and multiple nuclei arranged in a circle or hoarse shoe at the cell periphery. The diffuse granulomatous TB reaction was found in 7/16 (43.7%) of tuberculous cases and consisted of macrophages, lymphocytes, giant cells, fibroblasts, new capillaries and Langhans giant cells (Table-3).

Ziehl-Neelsen (ZN) stain:

Tubercle bacilli were seen inside the macrophages in the center of the granulomas.

Immunohistochemistry:

We found that immunoreactivity was cytoplasmic brown for iNOS (Fig. 3) and cytoplasmic light brown granular for CD68 (Fig. 4). Immunoreactivity for CD3 (Fig. 5) and CD20 (Fig. 6) were detected as membranous light brown staining. CD3 or CD20 was found to be expressed in the lymphocytes in the TB granuloma or diffuse TB reaction Table (3).

The macrophages in the granulomatous lesions were morphologically homogeneous in histological sections and were present in the center of the granuloma. Immunoreactivity to iNOS and the macrophage marker CD68 were intensely expressed in macrophage rich TB granuloma and in the diffuse granulomatous TB reaction consisting mainly of macrophages (epithelioid cells). Most Langhans cells (multinucleated giant cells) showed strong reactivity to both CD68 and iNOS. There were few iNOS and CD68 positive cells outside the TB reaction in the peritoneum. The expression intensity of iNOS and/or CD68 was stronger in diffuse and premature-stage granulomas than in late-stage granulomas (caseating granuloma).

The lymphocytes were usually found at the periphery of TB reaction with some dispersed cells in the center of the lesion. The CD3 (pan T marker), and CD20 (pan B marker) were localized to the lymphocytes. In TB granuloma, most $CD3^+$ cells were found at the periphery, and few $CD20^+$ cells in the center indicating typical TB granuloma. While, in diffuse TB granulomatous reaction $CD3^+$

lymphocytes were diffusely dispersed in the lesion with few $CD20^+$ lymphocytes.

Control cases of peritoneum removed with surgically excised organ specimens sent to our laboratory (e.g. with excised tumors) showed complete negativity for iNOS, CD3, very small number of CD68 and/or CD20 cells.

Table 1: Histological results of all patients

Histopathological results	n of patients
TB peritonitis	16
Malignant ascites	11
Bilharzial granuloma	1

Table 2: Clinical presentations of TB peritonitis patients (16 patients)

Clinical presentation	n of patients, (%)
Symptoms	
Fever	8 (50.0%)
Anorexia	7 (43.7%)
Weight loss	10 (62.5%)
Abdominal pain	8 (50.0%)
Abdominal swelling	7 (43.7%)
Cough	2 (12.5%)
Signs	
Abdominal tenderness	9 (56.3%)
Ascites	16 (100%)

Table 3: Histopathological and immunohistochemical results in tuberculous patients and controls

Tuberculous patients (n=16)		Controls (n=8)
Histopathology by H&E and ZN stain		
TB granuloma	9 (56.3%)	0
Diffuse granulomatous TB reaction	7 (43.7%)	0
Immunohistochemical staining		
iNOS (+ve)	16 (100%)	0
CD3 (+ve)	16 (100%)	0
CD68 (+ve)	16 (100%)	Few cells in 2/8 (25%)
CD20 (+ve)	16 (100%)	Few cells in 3/8 (37.5%)

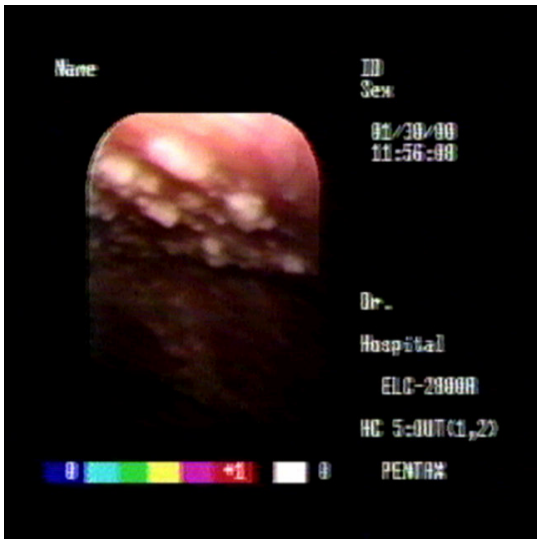

Figure 1: Laparoscopic picture of TB peritonitis showing tubercles on the parietal peritoneum.

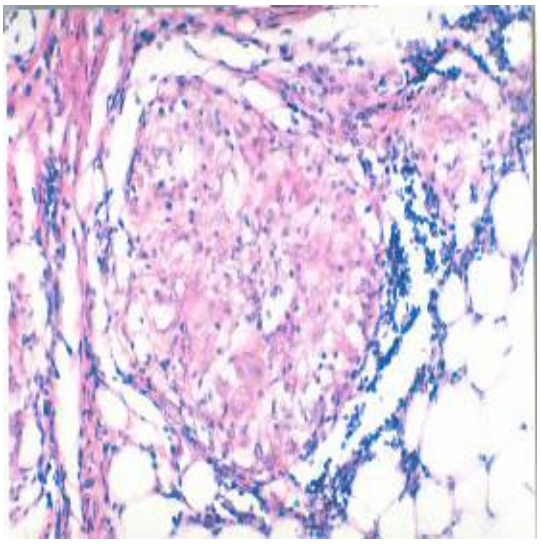

Figure 2: High power view X400 of H&E stained section showing TB granuloma in the peritoneum and diffuse TB reaction all around.

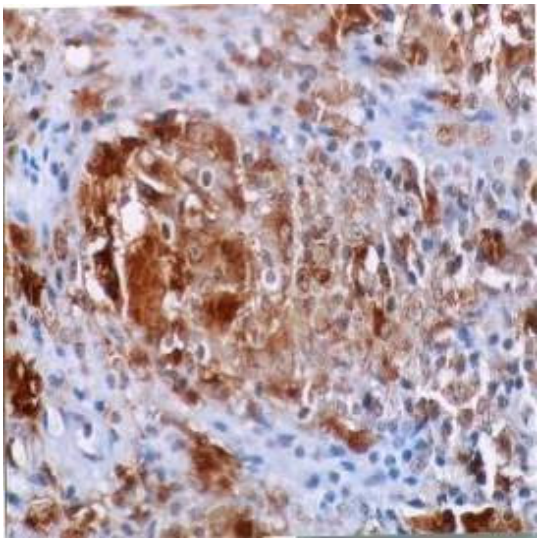

Figure 3: High power view X400 showing strong brown cytoplasmic expression of iNOS in TB granuloma in the peritoneum and also in the macrophages all around.

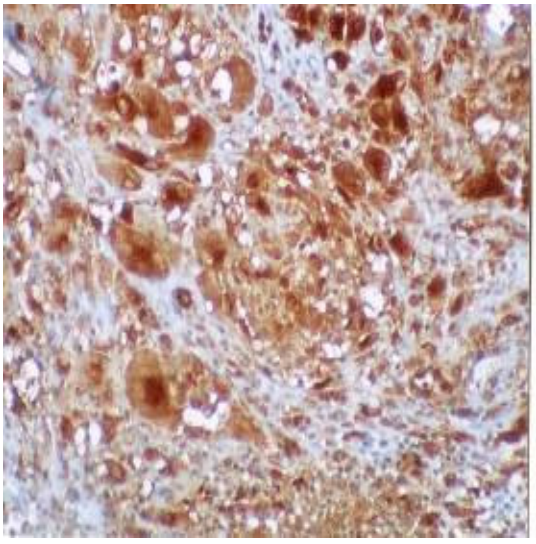

Figure 4: High power view X400 revealing strong cytoplasmic CD68 expression in macrophages in TB granuloma and in the surrounding diffuse TB reaction in the peritoneum.

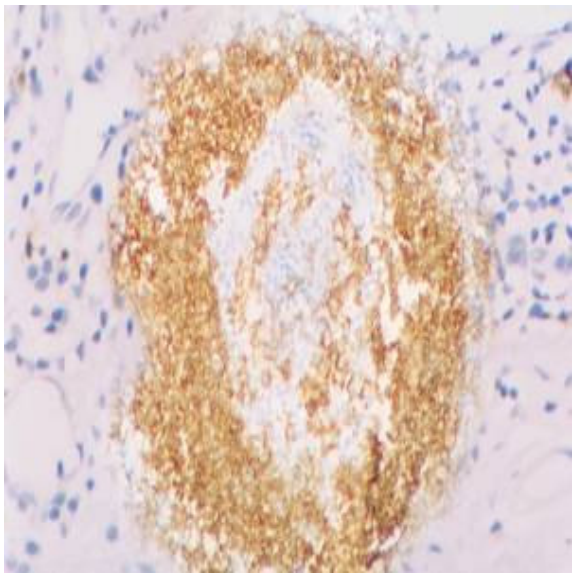

Figure 5: Tuberculous peritoneal granuloma showing CD3 positive lymphoid cells at its periphery (X400)

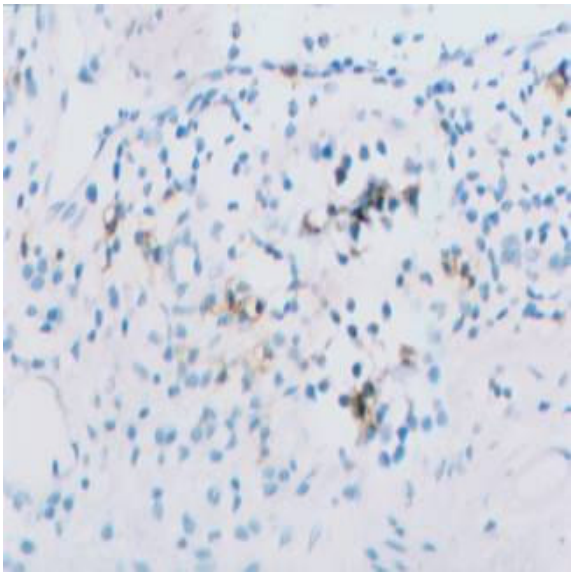

Figure 6: Tuberculous peritoneal reaction showing few CD20 positive lymphoid cells at its center (X400)

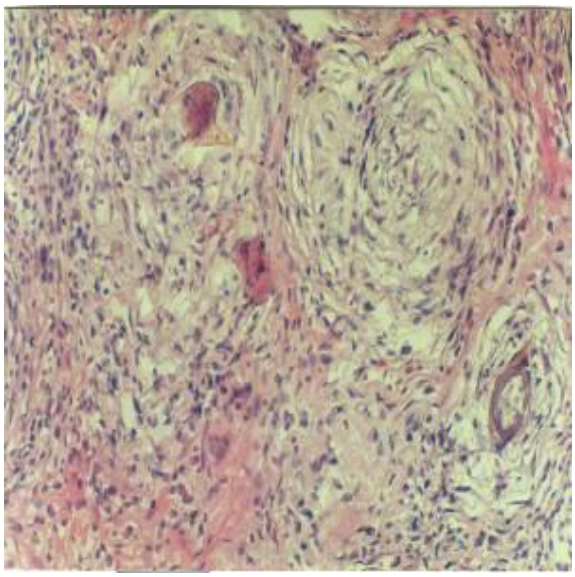

Figure 7: Bilharzial peritoneal granulomas by H& E stain with bilharzial ova in the center (X400)

DISCUSSION

Although many studies *in vitro* and animal models have been able to confirm an association between the production of NO and the killing of *M. tuberculosis,* the presence and role of NO production in human TB have been the subject of considerable disagreement [13, 27].

Nitric oxide (NO) is produced by a wide variety of cell types and is generated via oxidation of l-arginine that is catalyzed by the enzyme nitric oxide synthase (NOS). Nitric oxide synthase; NOS exists in three distinct isoforms: neuronal NOS (nNOS or NOSI), inducible NOS (iNOS or NOSII), and endothelial NOS (eNOS or NOSIII) [28].

Nitric oxide (NO) is a free gas radical that has been shown to have a number of important biological functions, including tumor cell killing, host defense mechanism against intracellular pathogens, vasodilatation, neurotransmission, inhibition of platelet aggregation, mediation of immune response, and participation in both acute and chronic inflammation [29].

The production of NO under oxidative stress conditions secondarily generates strong oxidizing agents (reactive nitrogen species) that may modulate the development of chronic inflammatory diseases and/or amplify the inflammatory response. The fundamental mechanisms driving the altered NO bioactivity under pathological conditions still need to be fully clarified, because their regulation provides a novel target in the prevention and treatment of chronic inflammatory diseases[28]. Pretreatment with nitric oxide synthase (NOS) inhibitors profoundly increases mortality, bacterial burden and pathological tissue damage in mice infected with *M. tuberculosis* [30].

Our finding of intense expression of iNOS in macrophage rich TB granulomas was in agreement with earlier studies showing that iNOS is present in alveolar macrophages-the first cells to encounter *M. tuberculosis*- in patients with pulmonary TB [31, 32]). Lymph nodes [33], pleural and pulmonary biopsies [22] from tuberculous patients showed expression of iNOS in granuloma associated macrophages. An increased NO metabolites have been observed in exhaled air of patients with active tuberculosis [31] and in tuberculous pleural effusion [34].

A potential therapeutic implication has been indicated by Schon et al.[35] where supplementation with arginine; the substrate for NO production, increased clinical improvement in patients with active TB during the intensive phase of anti-tuberculosis treatment, possibly by enhancing NO-mediated mycobacterial killing.

Pathologists have described the lung granuloma as the whole mark of pulmonary TB. Its morphology is characterized by a central necrotic core surrounded by concentric layers of macrophages, epithelioid cells, multinucleated Langhans giant cells, and lymphocytes [36]. Containment of *M. tuberculosis* at the site of primary infection by a cellular wall and a fibrotic outer layer prevents the pathogen from dissemination throughout the host and focuses the immune response to the site of mycobacterial persistence [21].

The order of cellular infiltration has been studied. Seiler et al. [37] and Ulrichs et al. [38] reported that neutrophils were the first cell type in both mouse model and tuberculous patients with lesions close to the chest wall. These were followed by macrophages. Lymphocytes formed the predominant cell type at later time points [38]. This order of infiltration suggested that the innate immune response forms the first line of defense resulting in necrosis, later replaced by the acquired immune response of cells, which encircle the necrotic core.

The present study showed that the macrophages in the granulomatous lesions were morphologically homogeneous in histological sections and were present in the center of the granuloma. Immunoreactivity to iNOS and the macrophage marker CD68 were intensely expressed in macrophage rich TB granuloma and in the diffuse granulomatous TB reaction consisting mainly of macrophages (epithelioid cells). Most Langhans cells (multinucleated giant cells) showed strong reactivity to both CD68 and iNOS. There were few iNOS and CD68 positive cells outside the TB reaction in the peritoneum. The expression intensity of iNOS and/or CD68 was stronger in diffuse and premature-stage granulomas than in late-stage granulomas (caseating granuloma) due to the presence of larger number of macrophages in the premature than in the caseating ones. These findings agree with a study conducted by Schon et al. [22] in which pleural, pulmonary and lymph node biopsies showed immunoreactivity to iNOS in macrophage rich granulomas identified by CD68 and in most of Langhans giant cells as well.

To the best of our knowledge, the current study is the first to describe the distribution of different lymphocytes in granulomas of TB peritonitis. The lymphocytes were usually found at the periphery of TB reaction with some dispersed cells in the center of the lesion. The CD3 (pan T marker), and CD20 (pan B marker) were localized in the lymphocytes. Most $CD3^+$ cells were found at the periphery, indicating typical TB granuloma. In TB granuloma, few $CD20^+$ cells were present in its center. While, in diffuse TB reaction $CD3^+$ lymphocytes were diffusely dispersed in the lesion with few $CD20^+$ lymphocytes.

Ulrichs et al. [38, 39] described the granulomatous reaction in human pulmonary tuberculosis. They reported by H&E stain an

overview of structural properties of the granulomas: central necrosis surrounded by distinct cellular layers forming concentric circles. $CD68^+$ antigen presenting cells (APCs) were identified by immuno-histochemistry in the inner layer surrounding central necrosis and in small aggregates within the peripheral lymphocyte aggregate composed of $CD4^+$, $CD8^+$, and B cells in active follicle-like centers resembling secondary lymphoid organs. They concluded that these follicles serve as the nucleus and active center for mycobacterial containment by the host immune response.

In conclusion, the structural properties and cell populations in human peritoneal tuberculous granuloma are basically the same as pulmonary TB granuloma. An increased expression of iNOS by macrophages in the center of the granuloma, but not in the healthy peritoneum indicates the presence of high-output NO production at this site of human tuberculosis. Further studies are needed to test the therapeutic implications of NO in different forms of TB.

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الملخص العربي

دراسة مناعية هيستوكيميائية لأكسيد النيتريك المحرض المخلق فى التورم الحبيبى الدرنى البريتونى

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لقد وجد أن أكسيد النيتريك الناتج عن أكسيد النيتريك المحرض المخلق مهم جدا فى دفاع العائل (الحامل) ضد ميكروب الدرن فى القوارض ولكن وجود ناتج كبير من أكسيد النيتريك فى الدرن البشرى (الذى يصيب الإنسان) لا زال مجال خلاف.

وتهدف هذة الدراسه الى

١) فحص تعبير أكسيد النيتريك المحرض المخلق "iNOS" فى الخلايا الآكلة الكبرى بالغشاء البريتونى فى حالات التدرن البريتونى .

٢) إلقاء الضوء على الخصائص التكوينية للدرن البريتونى .

تم الحصول على عينات نسيجية من الغشاء البريتونى من ثمانية وعشرين مريضا يعانون من استسقاء غير واضح السبب وقد تم فحص الأنسجة بصبغة الهيماتوكسلين والإيوسين وبناء على ذلك فأن العينات التى ثبت بها درن بريتونى تم صبغ قطاعات منها بالـــ iNOS المناعى الهيستوكيميائى وكذلك دليل الـ CD68 ودلائل الخلايا الليمفاوية من النوعين T, B وهما على التوالى CD3, CD20 وكذلك تم صبغ ثمانية عينات من الغشاء البريتونى لاستخدامها كمجموعة ضابطة .

تم تشخيص التدرن البريتونى فى ستة عشر مريضا . لقد وجد التورم الحبيبى الدرنى (الحبيبوم) فى ٩ حالات (٥٦%) أما التفاعل الناتج عن التدرنات الحبيبية المنتشرة فقد وجد فى الـ ٧ حالات المتبقية (٤٤%) وقد وجد أن التفاعل المناعى لـــ iNOS أو الدليل الخلايا الملتهمة الكبرى CD68 معبرا عنه فى الحبيبوم الدرنى الغنى بالملتهمات الكبرى وكذلك فى التفاعل الدرنى الحبيبى المنتشر وقد بينت خلايا Langhans (خلايه الدرن العملاقة متعددة الأنوية) تفاعلا قويا لكل من iNOS و CD68 . وقد وجد فى الحبيبوم أن الخلايا الايجابية الـ CD3 موجودة على المحيط مع وجود القليل من الخلايا إيجابية CD20 فى مركز الحبيبوم وقد أظهرت عينات المجموعة الضابطة خلوها تماما من iNOS أو CD3 وجود عدد قليل جدا من كل من CD68 ، CD20 .

ويمكننا أن نستنتج أنه فى حالات التدرن البريتونى التى لم يتم علاجها بعد وجدت زيادة فى تعبير الـ iNOS فى الخلايا الآكلة الكبرى بالحبيبوم دليلا على وجود زيادة فى إنتاج أكسيد النيتريك فى المرحلة النشطة من هذا النوع من الدرن . اننا فى حاجة إلى إجراء دراسات أخرى لاختبار التطبيقات العلاجية لأكسيد النيتريك فى الصور المختلفة لمرض الدرن .