Comparative Study of Cellular Immune Response In Cutaneous Blastomycosis

Dr. Azhar A. F. Al-attrraqhchi
Medicine College, University of Al-Nahrain /Baghdad
Email : tariq_963@yahoo.com
Hiba T. Hussain
Veterinary Medicine College, University of Baghdad/Baghdad
Haider Faisal
Medicine College, University of Al-Nahrain /Baghdad

Received on:11/1/2012 & Accepted on7/6/2012

ABSTRACT

Background: The immune response against *Blastomyces dermatiditis* is less clear than that of *Histoplasma* spp. or *Coccidioides* spp. This study was done to evaluate the cellular immune host response in patients with ulcerative and verrucous skin lesions.

Methods: This study was conducted on 50 patient attended Al-Kadhymiah Teaching Hospital/Dermatology Dep. in Baghdad (2009-2010). After processing of skin samples, all cases were studied for various lymphocyte subtypes by using direct dual immunofluorescence staining method. Independent sample t-test was used to make a comparison between groups and p-value <0.05 was considered significant.

Results: Histopathological examination showed 4 biopsies of skin lesions were *Blastomyces dermatitidis* positive and 46 biopsies were negative, two cases were ulcerative and two were verrocous . According to this classification, the total cell counts, CD3+ cells was found statistically different (p=0.034). Plasma cell (CD19), T-helper (CD4) and T-cytotoxic (CD8) also were significant when p values were (p=0.046), (p=0.020) and (p=0.018) respectively. According to the type of lesion whether it is ulcerative or verrocous, CD3+, CD4+, CD8+ and CD19+ counts for each type were significant when, P value were (p≤0.001, p=0.003, p=0.026 and 0.050) respectively. However, there were no significant difference was seen for these cells (p>0.05) among negative lesions.

Keywords: Immunity & Blastomycosis; Immune response of Cutaneous blastomycosis.

دراسة مقارنة للاستجابة المناعية الخلوية في داء الفطار البرعمي الجلدي

الخلاصة

الخلفية: إنّ المناعة الناتجة عن الاصابة بالفطار البرعمي اقل وضوحا من النوسجات أَو الفطار الكرواني. اجريت الدراسة لتقييم رَدِّ المضيّفِ المنيعِ الخلويِ في المرضى المصابين بآفات جلدية تقرّحيةِ وثالولية.

الطرق: أجريتْ هذه الدراسة على المرضى المراجعين لمستشفى الكاظمية التعليمي/ قسم الجلدية في بغداد (2008-2009). العينات دُرِستْ فيها انواع الخلايا اللمفية المُخْتَلِفة باستعمال طريقة

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التصبيغ المناعي المتألق المباشر الثنائيةِ. إختبار (تي-T) لعيّنةِ المستقلِ قد أُستَعملُ للمقارنة بين المجموعاتِ و قيمة ($p \leq 0.05$) إعتبرت هامّةاحصائياً .
النَتائِج: بينت النتائج النسيجية ان 4 مِن العينات كانت موجبة للفطار البرعمي و46 كَانتْ سلبية النتيجةُ. إعتمادا على هذا التصنيف، وُجِد ان عدد الخلايا اللمفية الكليّ مختلف بشكل إحصائي (p =0.034)، خلية بلازما ،الخلايا التائية المساعدة والخلايا الخلوية وُجِدَ هنالك فروقات هامّة احصائيا (p =0.046)، (p =0.020) و (p =0.018) على التوالي على مُقَارَنَة العينات الإيجابيةِ بالعينات السلبيةِ. الخلايا التائية و خلية بلازما ،الخلايا التائية المساعدة والخلايا الخلوية وجدت فروق هامّة احصائياً ($p \leq 0.001$, p=0.003 , p=0.026 و p=0.054) على التوالي عند المقارنةِ بين العينات الموجبة نسيجيا التقرّحيةِ والثالولية. وقد وجد انه لايوجد اي اختلاف احصائي بين الجروحِ السلبيةِ لكُلّ انواع الخلايا.($p > 0.05$)
الاستنتاج: من هذة الدراسة نستدل بانه ربما هنالك نوع اخر من الخلايا المسؤولة عن الدفاع ضد خلايا الفطارغير CD8+, CD4+ , كما ان خلايا CD4+ و CD8+ كانت سائدة في الافات التقرّحيةِ و الثالولية على التوالي في الافات الجلدية للفطار البرعمي.

INTRODUCTION

Blastomyces dermatitidis is found in moist soil and wood in the southeast United States, the Mississippi River valley, southern Canada, and Central America. Blastomycosis is also called Gilchrist's disease, Chicago disease, or North American blastomycosis The term "South American blastomycosis" is sometimes used to describe infection with Paracoccidioides brasiliensis(1, 2, & 3) blastomycosis causes clinical symptoms similar to histoplasmosis.(4). Infection appears to begin in the lung and, in acute cases, may resolve without spread to other organs, infection is usually progressive, either in the lung or in sites of hematogenous spread. Skin, bone, and the genitourinary tract are the most common sites of hematogenous dissemination (5).

You can get blastomyocosis by inhaling *Blastomyces dermatitidis* particles, a fungus that is found in moist soil, particularly where there is rotting vegetation. The fungus enters the body through the lungs, infecting them. The fungus then spreads (disseminates) to other areas of the body. The infection may affect the skin, bones and joints, and other areas. Blastomycosis is rare. It is most common in the central and southeastern United States, and in Canada, India, Israel, Saudi Arabia, and Africa.

Being around infected soil is the key risk factor. The disease usually affects people with weakened immune systems, such as those with HIV or who have had an organ transplant. Men are more likely to be affected than women (6).

Blastomycosis has been recently diagnosed in Iraq (7). The infection tends to be long and chronic with host mounting varied and sometimes unsuccessful immunological responses. Similar to other chronic infections, it is the cell mediated immune response that has critical importance in Blastomycosis Cellular immunity mediated by T lymphocytes, in particular *Blastomyces dermatitidis* (8). Cellular immune response mediated by antigen-specific T lymphocytes and lymphokine-derived macrophage's cell-mediated immunity plays a critical role in aborting fungal growth (9). B cells activation is markedly augmented by the co-receptor complex compressing three proteins CD21, CR2, and CD19. The immunoglobulin

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super family comprise molecules with structural characteristics similar to those of the immunoglobulin. This family include CD2, CD3, CD4, and CD8 (10).

In this study, we tried to compare between different lymphocyte subtypes (CD3, CD4, CD8 and CD19) in cutaneous blastomycosis, and to determine the role of each cell in different types of cutaneous blastomycosis.

MATERIALS AND METHODS

Fifty patients with clinically suggestive cutaneous blastomycosis were included in this study from patients attended Al-Kadhumyia Teaching Hospital and Baghdad Teaching Hospital / Dermatology Department (thirty one were males and nineteen were females) with verrucous and ulcerative lesions. The patients belong to heterogeneous populations in Baghdad city.

Samples collection were included skin biopsies from each patient, skin biopsies were taken from the site of lesion then divided into two parts, one put in 10% formalin for histopathology to make paraffin embedded tissue (blocks). The other were put in sterile normal saline and immediately cultivated on a suitable media (Sabouraud's Dextrose Agar, Himedia Company) (10).

Direct dual immunoflourescence staining of CD3, CD19, CD4, and CD8 were applied on tissue sections with thickness of 5 µm.

DIRECT DUAL-IMMUNOFLUORESCENCE PROCEDURE

Tissue sections have cellular antigens in which the flourochrome-labeled monoclonal antibodies are directed. Fluorescent microscope was used to detect fluorescently stained cells, indicating that specific antigens are detected by those monoclonal antibodies (11).

MATERIALS & EQUIPMENT

- Monoclonal fluorescence-labeled antibodies (anti-CD4and anti-CD8mAb combined, anti-CD3 and anti-CD19 mAb combined) (SeroTech. Company).
- Fluorescence microscope “Olympus”.

Procedure:-

1. De waxing and rehydration: Paraffin embedded sections were placed in a hot air oven at 65°C overnight, then dipped in xylene and ethanol containing jars as in the following orders:
   a. Xylene.
   b. Fresh xylene.
   c. Absolute ethanol.
   d. Ethanol (95%).
   e. Ethanol (70%).
   f. Ethanol (50%).
   g. Distilled water.

   All in which for five minutes.
2. For blocking the non-specific binding sites, 100 µl of a protein-blocking reagent was placed onto the section and incubated for 10 minutes in a

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humid chamber at room temperature. The slides were drained and blotted gently.

3. 50 µl of diluted primary antibody was placed onto each section and incubated for 1 hour at 37°C in a humid chamber. The slides were drained and blotted gently.
4. Slides were dehydrated by dipped in ascending concentration of ethanol and xylene containing jars as in the following order:
   a. Ethanol (50%).
   b. Ethanol (70%).
   c. Ethanol (95%).
   d. Absolute ethanol.
   e. Fresh xylene.

All in which for five minutes.

5. A drop of mounting medium (DPX) was placed onto each xylene-wet section then covered with a cover slip quickly.
6. Slides were viewed by the fluorescent microscope at X40 and X100 objective lenses, suitable countable fields were located, and by transmitted light the total lymphocytes were counted, then switched to the UV light and the only stained cells were counted. This maneuver was repeated frequently till 200 cells had been recovered and counted.
7. Calculation: Percentage of positively stained cells = (number of labeled cells) / (total number of cells) x 100%.

RESULTS

Culture results revealed that only 4 out of 50 patients (8%) were positive for blastomycosis, considering that four cases were ulcerative and four were verrocous.

Positive versus negative skin lesions and lymphocyte subtyping:

The total lymphocyte count (CD3+ cells) was statistically different (p=0.034) with higher percentage in histopathology positive form (73.4%) as compared with negative lesions (66.8%). T-helper (CD4) and T-cytotoxic (CD8) were also found to be significant (p=0.020) and (p=0.018) respectively in positive and negative lesions, when the percentages of positive to negative results were 34.75/ 57.38% for CD4 cells respectively, and 42.97%/65.86% respectively for CD8 cells. While, plasma cells (CD19+) showed no statistical significant difference (p=0.466), table (1).

Despite their decrease as compared with negative cases, the results showed that CD8+ (42.9%) was more than CD4+ cells (34.7%).

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Table (1): Descriptive statistical analysis in comparing between negative and positive blastomycotic skin lesions.

	Histopathology		**Sig. (2-tailed)**
	Negative (46)	Positive (4)	
CD3	66.861±7.936	73.400±9.747	0.034*
CD19	38.237±20.839	27.733±9.157	0.466^{NS}
CD4	57.382±21.530	34.733±24.736	0.020*
CD8	65.867±21.359	42.975±23.654	0.018*

Mean ± S.D.
*= Significant difference p value≤0.05

Ulcerative versus verrocous positive lesions and lymphocyte typing

In positive biopsies CD3+, was highly significant (p≤0.001) with higher mean in ulcerative lesion representing 79.6% when compared with verrocous lesions when the percentage was 60.9%. CD4+ was more in ulcerative lesions (43.6%) than this in verrocous ones (17%) with significant difference (p=0.003). The difference between CD8+ and CD19+ were significant, (p=0.026 and 0.050) in ulcerative and verrucouss lesions when the percentage of these cells of verrocous lesions were (39%) and (52.9%) respectively, when these of ulcerative lesions were (22.1%) and (57.3%) respectively, table (2).

Table (2): Descriptive statistical analysis of ulcerative and verrucous lesions.

	Type of lesion	**Mean ± S.D.**	**Significance**
CD3	Ulcerative	79.650±1.443	≤0.001**
	Verrucous	60.900±2.536	
CD19	Ulcerative	22.100±3.580	0.003*
	Verrucous	39.000±1.643	
CD4	Ulcerative	43.600±9.558	0.026*
	Verrucous	17.000±5.663	
CD8	Ulcerative	37.350±5.346	0.050*
	Verrucous	52.900±7.334	

*= Significant difference p value≤0.05
**= Highly significant difference p value≤0.001

DISCUSSION

The immunophenotyping of cellular subsets forms the basis of understanding the cellular immune response in various stages and in different variants of the disease like cutaneous leishmaniasis (12). Some work has been done to evaluate cellular immune host response in cutaneous blastomycosis by immunophenotyping of

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cellular subsets of T lymphocytes in peripheral blood as well as in skin tissues (13), but there where no such study has been conducted in Iraq before.

Our study revealed some striking similarities and differences when compared with earlier studies. A rich T cell component scattered amongst T cells and other cells was seen in all positive cases and it was statistically significant in comparison with negative lesions. The number of CD4+cells, CD8+ cells and CD19+ in our study were markedly lower in positive cases when compared with negative cases, and there was significant difference in CD4+ cells and CD8+ cells.

This difference indicates that there may be other cells form part of a chronic granulomatous infiltrate including the fibroblasts, histiocytes, macrophages, dermal dendretic cells and Langerhan's cells (14). The inflammatory cellular infiltrate in active lesions contained a large number of CD3 + cells but a low CD4+ and CD8+ cell count. This relatively low number of CD4+ and CD8+cells may be considered as an indirect indicator for the presence of the significant number of gamma delta, and/or CD45RO+ cells.

Th1-type cellular immune responses play a critical role in protection against infection with *Blastomycoces*. However, CD3+ T cells represents 79.6% and 60.9% in ulcerative and verrucous lesions respectively, it may revealed the presence of another subset of T cells (CD3+ but CD4- and CD8- T cells). These cells (gamma delta cells) are thought to play a direct role in the granulomatous reaction together with cytokine IL-1 and TNF alpha, possibly secreted by activated T lymphocytes (2). These cells seem to be the effectors cells in early stage of the disease and may be critical at the commencement of the immune response to *Blastomyces*.

CONCLUSIONS

From this study we concluded that there may another T-cell subset responsible for the immune response against *B.dermatitidis*, rather than CD4+, & CD8+. And CD4+ cells were predominant in ulcerative lesions, while CD8+ cells were predominant in verrucous ones.

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