STUDYING OF IMMUNE RESPONCE OF MICE IMMUNIZED BY *Rhizopus* ANTIGENES

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ABSTRACT

Zygomycosis is an infection causes by different fungi, *Rhizopus spp* are one of these causative fungi that ubiquitous in nature and have worldwide distribution. To find out the immune response against *Rhizopus spp* in this study two antigens were prepared from pathogenic *Rhizopus spp*. The first one was spore antigen which extracted from spores and the second was mycelium antigen from fungal mycelium. These antigens were used in experimental animals (30 mice) which were divided to three groups. The first group was immunized by spore antigen and the second group with mycelium antigen. The third group was a control. Immunological tests were performed for all tested groups including the delayed type hypersensitivity test to detect the cellular immunity. The results showed that the mean values of skin thickness in immunized mice with spore antigen at 24 hours post examination, in the 1st group were (1.7±0.07) while in the 2nd group were (2.4±0.14), and these values declined at 48 hours post-examination in the 1st group (1.1±0.04) and the 2nd group (1.7±0.06). The humeral immunity detected by Enzyme-linked Immunosorbent Assay (ELISA), the results showed that immunized animals with mycelium antigen express high levels in the means concentration of (IgG) (270.5±29.55) in the second group when compared with the first one (132.2±11.45). In the control group, (IgG) remained with normal range in all mice serum, was (8.1±1.3).

Key words: *Rhizopus*, spore antigens, mycelium antigens.

INTRODUCTION

Mucormycosis (phycomycosis, zygomycosis) is a rare opportunistic fungal infection caused by fungi belonging to the Mucorales order and the Mucoraceae family. It was first described by Paultauf in 1885 (Viterbo *et al.*, 2011)

Mucormycosis is one of the most rapidly progressing and fulminant forms of fungal infection which usually begins in the nose and paranasal sinuses following inhalation of fungal spores (Syeda *et al*., 2017). It is caused by organisms including genera as Absidia, Mucor, Rhizomucor and Rhizopus. The common form of this infection is seen in patients with immunocompromised

state such as diabetes (Syeda *et al*., 2017). The main clinical presentations include rhinocerebral, pulmonary, and cutaneous forms (superficial) and less frequently, gastrointestinal, disseminated, and miscellaneous forms (Castrejon *et al*., 2017).

Rhizopus is a cosmopolitan filamentous fungus isolated in soil, decaying fruit and vegetables, animal feces, and old bread. While *Rhizopus spp*. are common contaminants, they are also occasional causes of serious (and often fatal) infections in humans. Some species are plant pathogens (Sutton *et al*., 1998).

Rhizopus spp. are among the fungi causing the group of infections referred to as zygomycosis. Although the term mucormycosis has often been used for this syndrome, zygomycosis is now the preferred term for this angio-invasive disease. *Rhizopus arrhizus* is the most common cause of zygomycosis and is followed by *Rhizopus microsporus var. rhizopodiformis* (Chakrabarti *et al*., 2001).

MATERIALS AND METHODS

Activation of isolates

Colony of *Rhizopus spp*. was identified as grayish-brown, wooly, and covered the whole petri dish, back reverse appeared pale yellow.

Microscopically, after staining with one drop of lactophenol cotton blue stain showed *Rhizopus spp*. had hyaline hyphae, which were broad and aseptateand. The rhizoids also seen. The sporangiophores were long and straight. Sporangia with columellas lacked apophyses.

Reactivation of *Rhizopus spp* strain was done through:

1- The isolate which previously diagnosed, it was subculture on Sabouraud dextrose agar and incubated at 25 ºC for 3-4 days.

2- After that, the mould growth on Sabourauds dextrose agar was harvested by 5 ml phosphate buffered saline 7.2 solution and centrifuged at (2000 rpm for 10 minutes at room temperature), the sediment was taken and washed for three times by PBS 7.2 and then re suspended by PBS 7.2. The concentration of fungus suspension was adjusted to 1×10^7 cell ml^{-1} for *Rhizopus spp*. by using heamocytometer chamber and red blood cells count method (Daci *et al*., 1984).

3- 0.5 ml of fungus suspension (1×10^7) was injected intraperitoneally in two aged weak mice with poor feeding for 1-2 weeks, then killed and pure *Rhizopus spp* was re isolated from their internal organs and cultured on sabouraud dextrose agar which incubated for 3-5 days, then harvested by PBS 7.2 PH and

suspended and injected to other mice. This procedure was repeated for several times until a virulence strain was obtained.

4- The virulent strain was used for preparing antigens.

Preparation of antigens

The virulence strain of *Rhizopus spp* cultured in sterile universal tubes which contained 50 ml of sabouraud dextrose broth with chloramphenicol after that incubated at 25-27 ºC for 5 days, and after growth cultured in one later of sabouraud dextrose broth that incubated at 25-27 ºC for 3 days with continuous vibration by shaker to obtain larger amount of fungal growth.

Tow antigens were prepared from virulent *Rhizopus spp*. the fungal growth was filtrated by using sterile glass funnel contain several layer of sterile gauze to separate the spores and mycelium. Then 250 ml of suspension contain spores was taken and added to 750 ml ethanol 99% and kept at 4 °C for 24 hr for precipitation spores. The sediment washed twice with ethanol 99% and centrifuged at 2000 rpm for 10 minutes at 25 ºC.

The sediment was taken and distal water added and shake to dissolve sedimentation. The mixture was centrifuged at 2000 rpm for 10 minutes at 25 ºC to precipitate non soluble material.

The supernatant kept in sterile universal tube until used as spores antigens (SAg).

After spores separation the accumulations mycelium put it in sterile petri dish with acetone 95% in refrigerator for 24 hr. The mycelium dried by desiccators, then grind by sterile mortar.

The ether solution was added to remove fat layer and alkaline buffer solution PH=8 was added to extract the effective material and clarification by filter paper (Whatman No1) then sterilized by Millipore filter paper and kept in universal in freezing at -18 ºC until using as a mycelium antigen MAgs. (Alameed *et al*., 2008)

Experimental design

Thirty white Swiss BALB/C mice from both sexes were divided randomly into three groups as following:

1- 1st group G1: (n=10 mice) was immunized intrapretonialy (I/P) with 0.5 ml of SAgs (30 mg ml^{-1} protein concentration), and after two weeks intervals, a second dose was given.

2- 2nd group G2: (n=10 mice) was immunized intrapretonialy (I/P) with 0.5 ml of MAgs (27 mg ml^{-1} protein concentration), and after two weeks intervals, a second dose was given.

3-3ed group G3: (n=10 mice) was inoculated with 0.5 ml of sterile PBS 7.2 pH I/P and served as a control group.

At the 30th day post-immunization, skin test was done for cellular immunity examination, and all animals from tested and control group were sacrificed. Blood samples were collected and serum were seperated from these animals for humeral immunity examination.

Immunological tests

Delayed type hypersensitivity test (DTH)

This test was done according to (Hudson *et al.*, 1980). 0.1 ml of spores antigens of *Rhizopus spp* (SAgs) contained 0.5 mg ml^{-1} protein concentration was injected intradermally in the left hind footpad of 5 mice from G1 while their right hind footpad was injected intradermally by 0.1 ml PBS (pH=7.2). Other 5 mice of G2 were inoculated intradermally with 0.1 ml contained 0.5 mg ml^{-1} protein concentration of MAgs, and the right hind footpad of these mice inoculated intradermally with 0.1 ml PBS (Ph=7.2). Thickness of footpad in all animals was measured by vernier caliper after 24 and 48 hours post injection.

Enzyme-linked Immunosorbent Assay (ELISA) method

This test was carried out according to manufacturer (immunological consultents laboratory, Inc.)®

RESULT AND DISCUSSION

Immunological test

Results of skin test (DTH)

The result of DTH at 24 hr post examination with SAgs in the 1st group (1.7±0.07) were lower than those values in the 2nd group (2.4± 0.14), and these values declined at 48hr post-examination in 1st group (1.1±0.04) and 2nd group (1.7±0.06) (Table 1).

Table 1. Mean values of difference skin thickness (millimeter) in immunized mice against SAgs and MAgs at 24 and 48 hr post examination

Groups	mean± SE *	
	After 24 hours	After 48 hours
Group 1 immunized with SAgs	1.7±0.07	1.1±0.04
Group 2 immunized with MAgs	2.4±0.14	1.7±0.06
Control group injected with PBS 7.2	0	0

*SE: standard error.
LSD = 0.2012

Enzyme-linked Immunosorbent Assay (ELISA) test

The results showed that immunized animals with MAgs express high levels in the means concentration of (IgG) (270.5±29.55) as compared with the second one SAgs (132.2±11.45), while IgG in the control group remain with normal range in all mice serum, was (8.1±1.3) (Table 2).

On the other hand, the first and second groups showed highly significant differences values of IgG means concentrations at (P≤0.05) significant level as compared with the control group.

Table 3. Mean values of serum level of IgG µg ml^{-1} in immunized and control animals

Groups	Mean level of Ab titer(ng ml^{-1})
1st group (immunized with SAgs)	132.2±11.45
2nd group (immunized with MAgs on)	270.5±29.55
3ed group Control	8.1±1.3

The results of immunological test revealed that the MAgs and SAgs stimulated both cellular and humeral immune responses, since these antigens contain all fungal molecules, and the nature of these antigens are proteins which stimulate a good immune responses.

since DTH is the principle pattern of cell mediated immunity (Ramzi *et al*., 1994). Protein antigens stimulated immune cells such as macrophages, dendritic cells and T cells through activated Th1 cells that produce immune cytokines such as granulocyte macrophages colony stimulator factors (GM-CSF), these cytokines accelerate haemopoiesis in the early step of differentiation of myeloid cells, resulting in increased production of neutrophils, monocytes and eosinophils (Armitage, 1998).

INF-y activated and attract these cells to the site of Ags inoculation and leads to induration and increased in thickness of the skin (Mencacci *et al.*, 2000), the number of activated these dependent on the level of cytokines release from active immune cells, therefore DTH is considered as one form of cell mediated immunity. This evidence supports the idea that mentioned by (Abdul-Zahra, 2009) who reported that culture filtrated Cryptococococcus antigens are stimulated a better cellular immune response in immunized mice.

The results demonstrated variation in the mean value of skin thickness against SAgs and against MAgs, these may be due to the protein nature of MAgs which lead to aggregated large number of immune and non-immune cells at the site of inoculation. Pietrella *et al.*, (2002) demonstrated that immunized mice with *Cryptococococcus neoformance* mannoproteins generated a good DTH response.

These results are in consistence with (Alameed *et al*., 2008) who reported that among mean titer of antibody of different rabbits groups which were immunized with mycelium antigen gave high level compared to the spore antigens immunization or the mix antigens of *Aspergillus fumigates*.

This study was agreement with (Weig *et al*., 2001) who showed that the spores antigen extract of *A. fumegatous* stimulated immune system in the rabbits and increase IgG titer in the serum compared to control group by ELISA test.

The results showed that the highest antibody titer seen in the group immunized with mycelium antigen. The differentiation between humeral immunity levels caused by the variation in quantity and quality of antigenic strictures in these antigens which have ability to stimulate immune system to produce the antibodies (Kauffman *et al*., 1994).

CONCLUSION

The result showed that the mycelium antigens gives good and high cellular and humeral immune response comparing to spore antigens. This may support to produce a good vaccinations for peoples under high risk from fungi belonging to the Mucorales order.

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دراسة الاستجابة المناعية للفئران الممنعة بمستضد الـ Rhizopus

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المستخلص

مرض الزايكومايكوسز او الميوكرمايكوسس هو مرض فطري والمتسبب بمجموعة من الفطريات الواسعة الانتشار في الطبيعة من رتبة mucoral وبضمنها فطر الـ Rhizopus. ولمعرفة الاستجابة المناعية لفطر الـ Rhizopus في هذه الدراسة نوعين من المستضدات تم تحضيرها من العترة الضارية للفطر، المستضد البوغي المستخلص من ابواغ الفطر ومستضد الغزل الفطري المستخلص من الخيوط الفطرية للعفن. استخدمت في الحيوانات المختبرية (30 فأر) قسمت الى ثلاثة مجاميع الاولى؛ حقنت بالمستضد البوغي والثانية بمستضد الغزل والثالثة تم اعتبارها مجموعة السيطرة.

اجريت الاختبارات المناعية على المجاميع الثلاثة والتي شملت اختبار فحص الحساسية الجلدي المتاخر. بينت النتائج وجود زيادة معنوية في سمك (تثخن) الجلد بعد 24 ساعة (1.7±0,07) في المجموعة الاولى و(2.4±0,14) في المجموعة الثانية وبعد 48 ساعة كانت (1.1±0,04) و(1.7±0,06) في المجموعة الاولى والثانية تباعا كنتيجة اللاستجابة المناعية الخلوية في المجموعتين مقارنة بمجموعة السيطرة. اما المناعة الخلطية فقد اجري اختبار الاليزا واظهرت النتائج وجود معدل تراكيز عالي من الاجسام المضادة في المجموعة الثانية ثم الاولى (270.5±29.55) و(132.2±11.45) وبمستوى معنوي عالي مقارنة بمجموعة السيطرة وعند مقارنة معدل تراكيزها في المجموعتين وجد ان مستضد الغزل الفطري قد اعطت اجسام مناعية عالية مقارنة بالمستضد البوغي.

الكلمات المفتاحية: الاستجابة المناعية، مستضد الـRhizops، الفئران.