# Effect of immunosuppression on the pathogenesis of respiratory zygomycosis in rabbits intranasally infected with *Absidia corymbifera*

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*Summary*

The study was envisaged for elucidating the effect of immunosuppression on the pathology and pathogenesis of experimental respiratory absidiomycosis in rabbits. Seventy rabbits used in the experiment were divided into different groups viz. non-immunosuppressed, immunosuppressed with cyclophosphamide and immunosuppressed with methylprednisolone respectively, and a control group. The experiment was continued for 50 days during which all the infected animals exhibited clinical signs of respiratory distress but no mortality. The infected rabbits showed gross lesions mainly in the form of pneumonic consolidations in the lungs in antero-ventral lobes initially followed by spread to the other lobes. Similarly, microscopic lesions were restricted to lungs and consisted of early pyogranulomas dominated by fungal invasion and spread, followed by chronic granulomatous reaction and a recovery during the later stages. Fungus was demonstrated and re-isolated only from lung lesions up to 15 days postinfection (DPI) in non-immuno-suppressed group and up to 30 DPI in both the immunosuppressed groups suggesting no systemic dissemination. The findings of the present study indicated that immunosuppression increased the extent, duration and severity of the pathological lesions associated with absidiomycosis in the lungs. Moreover, an assessment based on gross and histopathological lesions revealed cyclophosphamide to be a more potent immunosuppressant than methylprednisolone.

*Key words*

Zygomycosis, Rabbits, *Absidia corymbifera*, Pathology, Immunosuppression, Cyclophosphamide, Methyl-prednisolone

## Efecto de la inmunosupresión en la patogenia de la zigomicosis respiratoria en conejos infectados intranasalmente con *Absidia corymbifera*

*Resumen*

El propósito de este estudio fue dilucidar el efecto de la inmunosupresión en la patología y patogenia de la absidiomicosis respiratoria experimental en conejos. Los 70 conejos utilizados en el experimento se dividieron en los siguientes grupos: no inmunosuprimidos, inmunosuprimidos con ciclofosfamida, inmunosuprimidos con metilprednisolona y grupo control. Durante los 50 días que duró el experimento, todos los animales infectados mostraron signos clínicos de disfunción respiratoria pero no hubo mortalidad. Los conejos infectados presentaron amplias lesiones fundamentalamente en forma de consolidaciones neumónicas inicialmente en los lóbulos anteroventrales de los pulmones seguidas de diseminación a los otros lóbulos. De igual modo, las lesiones microscópicas se restringieron a los pulmones y consistieron en piogranulomas tempranos dominados por invasión fúngica y diseminación, seguida de una reacción granulomatosa crónica y de una recuperación en los estadios tardíos. Sólo se pudo demostrar y reaislar el hongo de las lesiones pulmonares hasta 15 días después de la infección en el grupo de conejos no inmunosuprimidos y hasta 30 días en ambos grupos inmunosuprimidos, sugiriendo la inexistencia de diseminación sistémica.

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Los hallazgos de este estudio indican que la inmunosupresión produjo un aumento de la extensión, duración y severidad de las lesiones patológicas asociadas con la absidiomicosis pulmonar. Por otro lado, el seguimiento de las lesiones macroscópicas e histopatológicas reveló que la ciclofosfamida es un inmunosupresor más potente que la metilprednisolona.

*Palabras clave* Zigomicosis, Conejos,*Absidia corymbifera*, Patología, Inmunosupresión, Ciclofosfamide, Metilprenisolona

Respiratory zygomycosis affects both humans [1] and animals [2]. In the current era of widespread use of broad spectrum antibiotics, corticosteroids, cytotoxic agents and immunosuppressants, besides increasing incidence of acquired immunodeficiency syndrome (AIDS) and diabetes coupled with stressful life styles, fungi of the class zygomycetes, once thought to be non-pathogenic, are emerging as agents of serious respiratory and systemic infections and have therefore gained considerable public health importance [3,4]. In a previous communication [5], we reported the pathogenesis of experimental respiratory zygomycosis in the non-immunosuppressed Asian water buffaloes. This paper deals with the effect of immunosuppression on the pathology and pathogenesis of experimental respiratory zygomycosis in rabbits.

## MATERIAL AND METHODS

*Experimental animals.* Seventy male New Zealand-White rabbits (*Oryctolagus cuniculus*) aged 6-8 months were kept under observation in thoroughly cleaned rooms for 15 days prior to starting the experiment. Food and water were given ad libitum. The animals were immunologically normal and their sera were negative for *Absidia corymbifera* antibodies by agar gel precipitation test (AGPT).

*Fungal strain and preparation of inoculum.* A strain of *A. corymbifera* (MTCC No. 379) obtained from the Institute of Microbial Technology, Chandigarh, India, was used. The isolate was grown on Sabouraud dextrose agar (SDA) containing 0.3% chloramphenicol. After incubation for 3-4 days at 37∞C, the culture was flushed with sterile phosphate buffered saline (PBS, pH 7.4) containing 0.05% Tween-80. The sporangiospore suspension so obtained, was shaken for 2h on a mechanical shaker and sporangiospore concentration was adjusted to 1.4 x $10^5$/ml using a haemocytometer.

*Design of experiment.* The animals were randomly divided into three infected groups consisting of 20 rabbits each and one control group comprising 10 rabbits. Of the two infected groups one was immunosuppressed with cyclophosphamide (Group C) (Endoxan; German Remedies, India) and the other with methylprednisolone (Group M) (Solu-Medrol; Upjohn, India), given at the dose rate of 10 mg/kg body weight (b.wt.) for 5 days prior to infection followed by 5 mg/kg b.wt. for another 5 days after the infection. The 3rd infected group received no immunosuppressive therapy (Group A). Each animal in all three infected groups was intranasally administered 1 ml suspension of *A. corymbifera* containing 1.4 x $10^5$ sporangiospores. The animals of the control group were similarly administered 1 ml of sterile PBS containing 0.05% Tween-80.

The animals of all four groups were kept in separate and distantly located rooms. The control animals were always attended, fed and watered before handling the infected animals or contaminated material. The animals were closely observed daily for clinical signs, if any.

The experiment was continued for 50 days during which two randomly selected animals from each of the infected groups and one animal from the control group were killed at 1, 2, 3, 5, 10, 15, 20, 30, 40 and 50 days post infection (DPI) and subjected to necropsy. Gross changes, if any, were recorded and slices from lungs, liver, kidney, brain, spleen and trachea were fixed in 10% buffered formol saline for histopathology. Paraffin sections, 5 mm thick, were cut and stained with haematoxylin and eosin (H&E). For demonstration and confirmation of fungi in tissues, sister sections were also stained with the periodic acid Schiff (PAS), Grocott's methenamine silver nitrate (GMS), combined GMS-HE and indirect immunoperoxidase [6-8]

*Re-isolation of fungus.* Re-isolation of *A. corymbifera* was attempted from blood, liver, kidney and lung tissue cultured on SDA slants incubated at 37∞C for 2 to 7 days.

## RESULTS

*Clinical signs.* Rabbits in all the three infected groups showed dullness, depression and mucopurulent nasal discharge during the first 10 days of the experiment. Additionally, infected rabbits in groups C and M showed partial anorexia and initial pyrexia during the first week after infection. However, there was no mortality. The control animals did not show any clinical signs.

*Gross lesions.* Gross lesions were recorded mainly in the lungs of the animals in all the three infected groups and were most extensive and severe and persisted for longer period in group C animals, followed by groups M and A (Figures 1-3). In the first two days, there were areas of red hepatization in the anteroventral lobes. From DPI-3 onwards, the pneumonic consolidations became greyish and more extensive involving anterior parts of diaphragmatic lobes also. From DPI-10 onwards, the lesions became nodular, more extensive and involved almost all the lobes and their cut surfaces revealed caseated pus. On DPI-20 in group A, the lungs did not show any pathological change, whereas, in groups C and M, the gross lesions continued up to DPI-30 and the lungs in these groups also became apparently normal by DPI-40. In addition, trachea showed congestion in all the three infected groups.

*Histopathology.* The mycotic lesions were restricted only to the lungs, the reaction being most severe in group C, followed by groups M and A, respectively.

On DPI-1, clumps of sporangiospores of *A. corymbifera* were seen in the lumina of bronchioles and air spaces. Inflammatory cell reaction was acutely purulent, characterized by infiltration of neutrophils around the sporangiospores (Figure 4) and fungal hyphae in the lung parenchyma. On DPI-2 and 3, lungs showed mainly pyogranulomas with fungal elements in their centers, surrounded by eosinophilic Splendor-Hoeppli bodies (Figures 5 and 6) and in places giant cell formation. On DPI-5, pyogranulomatous reaction and fungal infection were more severe than those on earlier days. Besides that, there was

Figure 1. Cut surfaces of the lung of group C animal (DPI-10), showing most extensive greyish mycotic lesions.

Figure 2. Cut surfaces of the lung of group M animal (DPI-10) showing less severe and less extensive greyish mycotic lesions than seen in group C animal.

Figure 3. Cut surfaces of the lung of group A animal (DPI-10) showing least extensive mycotic lesions.

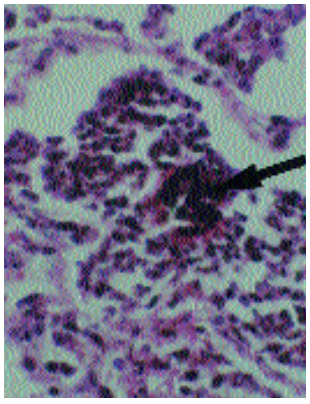

Figure 4. Lung (DPI-1). Clumps of sporangiospores of *A. corymbifera* (arrow), surrounded by neutrophilic infiltration. GMS-H&E X 300.

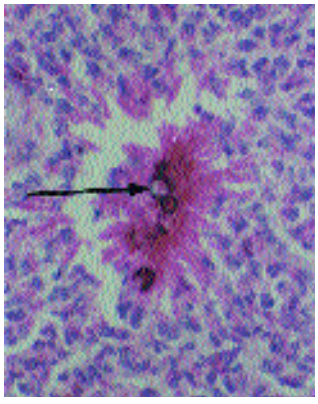

Figure 5. Lung (DPI-3). Pyogranuloma with fungal elements in the centre (arrow), surrounded by eosinophilic Splendor-Hoeppli bodies. GMS-H&E X 300

marked macrophage, lymphocyte and giant cell infiltration along with thick peribronchiolar and perivascular lymphocytic cuffing. Phagocytosis of fungal elements by giant cells was also very prominent (Figures 7 and 8). In animals of group C, fungal invasion in the lungs was most

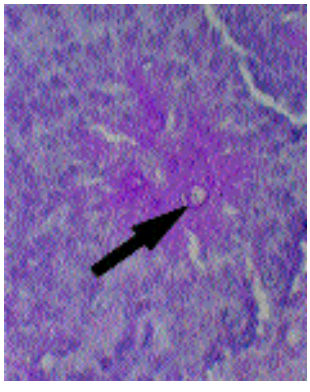

Figure 6. Same as in Figure 5 in PAS stain X 300.

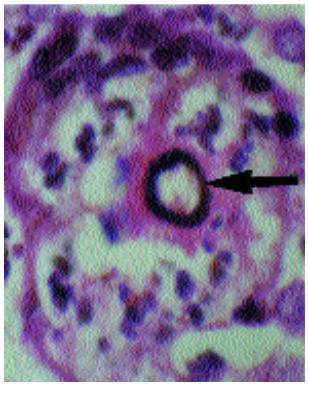

Figure 7. Lung (DPI-5). Phagocytosed fungal element (arrow) in foreign body giant cell. GMS- H&E X 1000.

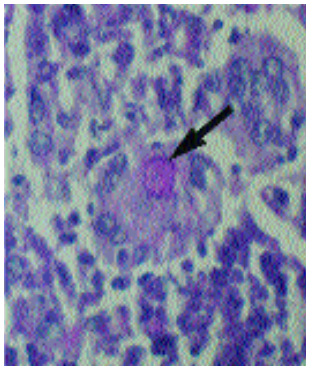

Figure 8. Same as in figure 7 by PAS stain x 300.

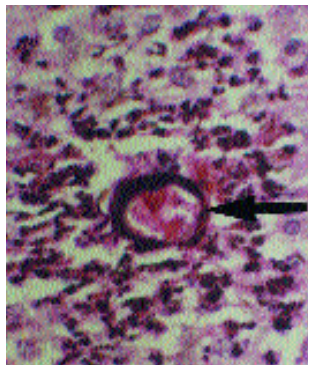

Figure 9. Lung of group M animal (DPI-5). Severe neutrophilic reaction around the fungal element (arrow). GMS- H&E X 300.

severe and in group M neutrophil infiltration around the fungal elements was more conspicuous (Figure 9). By DPI-10, foci of necrosis were observed in the lungs of all the animals in group C (Figure 10).

At DPI-15, in group A, the fungal elements in the form of disintegrated granules were phagocytized by the giant cells and macrophages (Figure 11). The lungs of group C animals at this stage showed the most severe reaction in the form of variable sized areas of caseative necrosis containing fungal elements both within and outside. At DPI-20, lungs of group A animals showed only

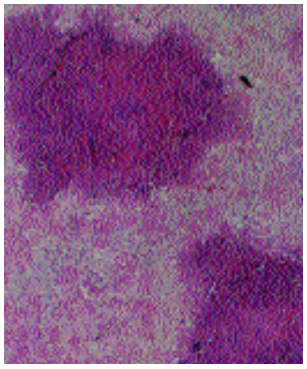

Figure 10. Lung of group C rabbit (DPI-10). Foci of caseative necrosis. GMS-H & E X 70.

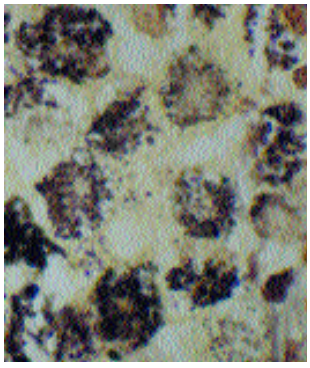

Figure 11. Lung of group A animal (DPI-15). Disintegrated fungal granules in the macrophages. GMS X 1000.

interstitial pneumonia and lymphocytic infiltration, but no pyogranulomas and fungal elements, whereas, groups C and M animals showed more extensive caseative necrosis containing fungal elements than that seen on DPI-15. From DPI-30 onwards, the lungs of group A animals became normal, whereas, the lungs of the animals in the groups C and M still showed pyogranulomatous reaction with fragments of fungal material in their centers. Animals in the control group did not show any gross or histopathological lesion at any interval.

*Re-isolation of the fungus. A. corymbifera* was consistently re-isolated only from the lung lesions in non-immunosuppressed group A from DPI 1 to 15 and in immunosuppressed groups from DPI 1 to 30. Fungus was not re-isolated from any organ in the control group.

## DISCUSSION

The clinical signs in this study were very similar to those recorded in naturally occurring zygomycosis in sheep and goats [9,10] and experimentally induced zygomycosis in buffalo calves [5].

The observations regarding the confinement of gross mycotic lesions to antero-ventral lobes of the lungs initially and then spreading further in other lobes were similar to those in experimental absidiomycosis in buffalo calves [5] and experimental aspergillosis in goats [11,12]. The reason, the extrapulmonary organs were not significantly affected might be due to the considerable effects of germinating inhibitors and the concentration of the basic proteins present in such tissues [13]. However, increased severity and extent of gross lesions in immunosuppressed animals indicated greater fungal spread and invasion [11,14-17], although Corbel and Eades [18] contradicted the role of cyclophosphamide in increasing the susceptibility of mice to absidiomycosis.

The early inflammatory response characterized by marked infiltration of neutrophils might have been invoked by physical irritation due to fungal spores [19]. Similar changes were recorded in experimental absidiomycosis [5] and experimental aspergillosis [11,12] in animals. The pyogranulomatous reaction as observed at different intervals indicated the role played by both macrophages and neutrophils in defenses against fungi [20]. However, the Splendore-Hoeppli phenomenon around the fungal elements may be regarded as a typical characteristic of zygomycosis [21]. Similar lesions and reaction had earlier been recorded in experimental intratracheal absidiomycosis in buffalo calves [5]. The granulomas composed of macrophages, giant cells, lymphocytes, plasma cells and epithelioid cells as also recorded in the study are known to occur as an allergic response to fungal infection in humans [22-24]. Cyclophosphamide was found to be a more potent immunosuppressant than methyleprednisolone as the extent and severity of fungal invasion increased in the rabbits treated with the former, although the latter appeared to invoke a greater neutrophilic response. Similar findings were also reported by others [15,25]. The minimal lymphocytic infiltration in pulmonary lesions of methylprednisolone treated rabbits may well be due to potent antilymphocytic activity of the drug [15,17,25]. The other tissue responses like caseative necrosis, giant cell formation and interstitial pneumonia with simultaneous clearance of fungal elements also lasted longer in the immunosuppressed rabbits than non-immunosuppressed ones, simulating such observations made by several other authors [5,14,16,18,26]

On the basis of gross and histopathological lesions coupled with prolonged re-isolation of the fungus in immunosuppressed groups (up to DPI-30) when compared to non- immunosuppressed groups (up to DPI-15), it may be safely inferred that immunosuppression increased the extent, severity and duration of lesions induced after intranasal administration of *A. corymbifera* in rabbits. In addition, the self-limiting nature of the absidiomycosis that we observed in the non-immunosuppressed animals, has been well documented [5,26].

Our findings in experimental absidiomycosis differed significantly from those of Corbel *et al.* [27] and Sodhi *et al.* [28] who observed mycotic gross and microscopic lesions only in the kidneys rather than lungs. These differences may be due to the fact that the intravenous route of infection was used instead of the intranasal or intratracheal one.

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