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Effect of Immunosuppression on the Clinicopathological Changes in Experimental Zygomycosis in Rabbits

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ABSTRACT

A study was undertaken to investigate the effect of immunosuppression by cyclophosphamide or methylprednisolone on the clinicopathological alterations in respiratory absidiosis in rabbits. Infected rabbits showed respiratory distress that was more severe in immunosuppressed groups. Leukocytosis due to neutrophilia was observed in the non-immunosuppressed group in the initial stages, whereas leukopenia was observed in both the immunosuppressed groups initially, owing to polymorphopenia in the cyclophosphamide-treated group and to lymphopenia in the methylprednisolone-treated group, followed by leukocytosis in both groups. Total serum proteins increased significantly in the non-immunosuppressed group but were significantly decreased in the immunosuppressed groups. Serum creatinine increased significantly in all the infected groups from 20 days post inoculation (DPI) onwards. Blood urea nitrogen increased significantly in the initial stages only in the methylprednisolone-treated group. AST and ALT also showed significant increases in the infected animals. Total serum immunoglobulins and circulating immune complexes increased gradually in all three infected groups, except for an initial significant drop in the immunosuppressed rabbits. Re-isolation of fungus was only achieved from the lungs of infected rabbits up to 15 DPI in the non-immunosuppressed group and 30 DPI in the immunosuppressed groups. Pathological lesions in all the infected groups were found mainly in the lungs and consisted of pyogranulomas. The lesions were most severe in the cyclophosphamide-treated group and least severe in the non-immunosuppressed group.

Keywords: Absidia corymbifera, creatinine, cyclophosphamide, leukocytosis, lung, methylprednisolone, rabbit, serum proteins

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CICs, circulating immune complexes; DLC, differential leukocyte count; DPI, days post inoculation; GMS, Grocott's methanamine silver nitrate; Hb, haemoglobin; IIP, indirect immunoperoxidase; Ig, immunoglobulin; H&E, haematoxylin and eosin; PAS, periodic acid–Schiff; PBS, phosphate-buffered saline; PCV, packed cell volume; SDA, Sabouraud's dextrose agar; TLC, total leukocyte count

INTRODUCTION

Respiratory zygomycosis is rapidly gaining importance as a public health problem in humans (Iwen *et al.*, 1997) because of the widespread and prolonged use of antibiotics, corticosteroids, and cytotoxic and other immunosuppressant drugs, coupled with an increasing incidence of autoimmune and immunodeficient diseases (Mackenzie *et al.*,

1988; Christopher *et al.*, 1989; Gaing *et al.*, 1992; Hyatt *et al.*, 1992; Nagao *et al.*, 1993; Rodriguez *et al.*, 1993; Lim *et al.*, 1994; Hicks *et al.*, 1995; Mohsenipour *et al.*, 1995; Janaki *et al.*, 1998; Oberai, 1998). Therefore, the effect of immunosuppression in influencing the severity and outcome of fungal diseases, such as zygomycosis, needs to be studied in detail. However, only a few experiments have been conducted to study the role of immunosuppression in experimental zygomycosis in animals (Baker and Linares, 1974; Corbel and Eades, 1975; White *et al.*, 1978; Kitz *et al.*, 1981; Waldorf *et al.*, 1983). Gupta and colleagues (1999) reported the haematological, biochemical, immunological and pathological changes in non-immunosuppressed buffalo calves following intravenous inoculation with *Absidia corymbifera* spores. The effects of the immunosuppressants, cyclophosphamide and methylprednisolone, on the clinicopathological parameters in experimental respiratory zygomycosis were studied in rabbits.

MATERIALS AND METHODS

Experimental animals

Seventy male New Zealand White rabbits (*Oryctolagus cuniculus*), aged 6–8 months, were kept under observation for 15 days before starting the experiment. The animals were immunologically normal and their sera were negative for *A. corymbifera* antibodies as determined by the agar gel precipitation test, using a soluble antigen prepared by the method of Khan and colleagues (1976). Before housing the animals, the experimental rooms were thoroughly cleaned with 2.5% phenol and subsequently fumigated with formaldehyde gas. Food and water were given *ad libitum*.

Fungal strain and preparation of inoculum

A standard strain of *A. corymbifera* (MTCC no. 379) was obtained from the Institute of Microbial Technology, Chandigarh, India. The isolate was grown on Sabouraud's dextrose agar (SDA) containing 0.3% chloramphenicol. After incubation for 3–4 days at 37°C, the growth was flushed with sterile phosphate-buffered saline (PBS, pH 7.4) containing 0.05% Tween-80. The spore suspension was shaken for 2 h on a mechanical shaker and the concentration was determined and adjusted to 1.4×10^5 spores/ml using a haemocytometer.

Experimental design

The animals were randomly divided into three infected groups of 20 rabbits each and one control group of 10 rabbits. Two infected groups were immunosuppressed with cyclophosphamide (group C) (Endoxan, German Remedies, India) or methylprednisolone (group M) (Solu-medrol, Upjohn), respectively, at a dose of 10 mg/kg body weight for 5 days prior to infection followed by 5 mg/kg body weight for another 5 days after

infection. The third infection group (group A) received no immunosuppressive therapy. The rabbits in all three infected groups received 1 ml of suspension of *A. corymbifera* containing 1.4×10^5 spores delivered intranasally. The 10 animals in the control group similarly received 1 ml of sterile PBS containing 0.05% Tween-80.

The animals in the different groups were kept in separate and distantly located rooms. The control animals were always attended, fed and watered before the infected animals or contaminated material were handled. The animals in all the groups were closely observed daily for clinical signs. The experiment was continued for 50 days, two animals chosen at random from each infected group and one from the control group being killed on days 1, 2, 3, 5, 10, 15, 20, 30, 40 and 50 after infection.

For haematological, biochemical and immunological studies, blood samples were collected from all the infected and control animals 0, 5, 10, 15, 20, 30, 40 and 50 days post infection (DPI). For haematological studies, 5 ml of blood was collected into vials containing dipotassium ethylenediaminetetraacetate, using 2 mg of powder for 1 ml of blood. For biochemical and immunological studies, the blood was collected into sterilized tubes for serum separation. Haemoglobin (Hb), packed cell volume (PCV), total leukocyte counts (TLC) and differential leukocyte counts (DLC) were conducted by the methods of Jain (1986).

The biochemical studies included estimations of total serum protein, serum albumin, blood urea nitrogen (BUN), serum creatinine, serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST). The values were determined using commercial kits and procedures procured from Bayer Diagnostic India Ltd, Baroda, India, the optical densities being read on an autoanalyser (Beckman Clinical System 700, The Netherlands).

The immunological studies included determination of total serum immunoglobulins (Oser, 1965) and circulating immune complexes (CICs) (Creighton *et al.*, 1973). The soluble antigen of *A. corymbifera* was prepared by the method of Khan and colleagues (1976) and hyperimmune serum was raised in New Zealand White rabbits by the method of Azuma and colleagues (1969).

Histopathological studies

Tissue slices, 1–2 cm thick, taken from different organs, were formalin fixed, paraffin embedded, sectioned at 5 μm and stained with hamatoxylin and eosin (H&E), combined Grocott's-H&E, Grocott's methanamine silver nitrate (GMS), periodic acid–Schiff (PAS), and indirect immunoperoxidase (Luna, 1968; Chandler, 1992; Sehgal, 1992).

Re-isolation of fungus

At necropsy, tissue samples from affected areas of the lungs, kidneys and brain were subjected to re-isolation of the fungus. The tissues (1–2 cm thick) were collected into sterile containers, cultured in Sabouraud's broth and later streaked on SDA medium and examined for fungal growth after incubation at 37°C for 2–7 days. The isolated *A. corymbifera* was identified and confirmed in the Department of Mycology of our college.

The lungs and blood samples were also subjected to bacteriological examination in the Department of Veterinary Microbiology at our college.

The results were subjected to statistical analysis by one-way analysis of variance (Gupta, 1985).

RESULTS

Clinical signs

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

Haematological studies

The Hb and PCV did not change appreciably from their basal values at any time in any of the infected animals in any of the three groups. The TLC was significantly elevated from 5 to 20 DPI in group A. In groups C and M, the TLC was significantly lower on days 0 and 5, but significantly raised at 20 and 30 DPI. Absolute leukocyte counts revealed a significant neutrophilia (heterophilia) from 5 to 20 DPI in group A. There was no significant alteration in the lymphocyte counts. In group C, there was a significant neutropenia on days 0 and 5 but a brief significant increase at 20 DPI. Lymphocytes were significantly reduced on days 0 to 20 and then increased from 20 DPI onwards. In group M there was a significant lymphopaenia on days 0 to 10, along with a neutrophilia on days 0 to 20. Thereafter, the lymphocytes increased steadily and lymphocytosis was observed at 20 and 30 DPI. No significant alterations were observed in the eosinophil, monocyte or basophil counts in any of the infected groups at any stage.

There were no significant haematological variations in any of the control animals at any stage.

Biochemical studies

In group A, the total serum proteins (Figure 1) were significantly raised from 5 DPI. In groups C and M, the total serum proteins were significantly decreased over days 0–20 DPI and days 0–30 DPI, respectively. However, the serum albumin concentration did not show any significant change in any of the groups. In groups A and M, serum creatinine (Figure 2) was significantly increased from 30 DPI onwards and in group C from 20 DPI onwards. In group M, BUN (Figure 3) was significantly raised over days 0–10, but there were no significant alterations in BUN in groups A and C. In groups A, C and M, AST (Figure 4) was significantly increased at 5–30 DPI. ALT was also significantly raised for short periods between 0 and 20 DPI in all three infected groups (Figure 5). There were no significant biochemical alterations in the control animals.

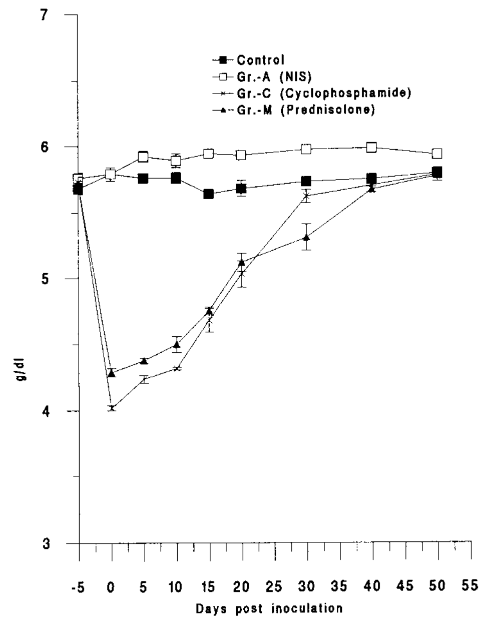

Figure 1. Changes in total proteins in the serum of rabbits intranasally infected with *Absidia corymbifera*

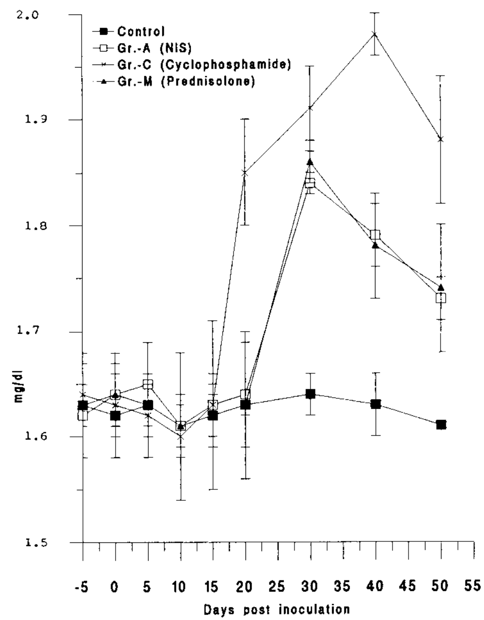

Figure 2. Changes in the creatinine concentration in the serum of rabbits intranasally infected with *Absidia corymbifera*

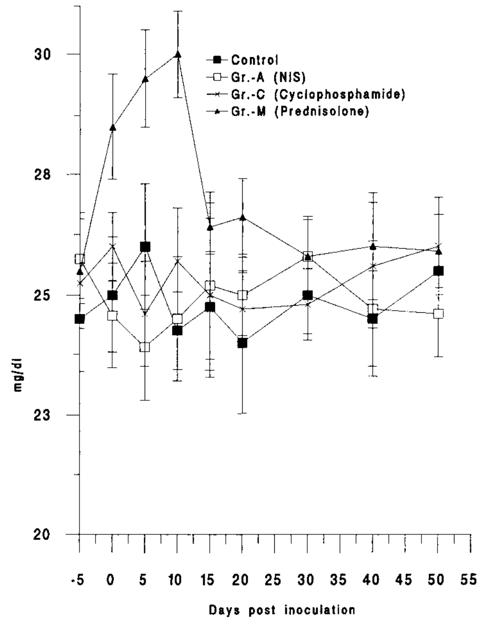

Figure 3. Changes in the blood urea nitrogen (BUN) concentrations in the serum of rabbits intranasally infected with *Absidia corymbifera*

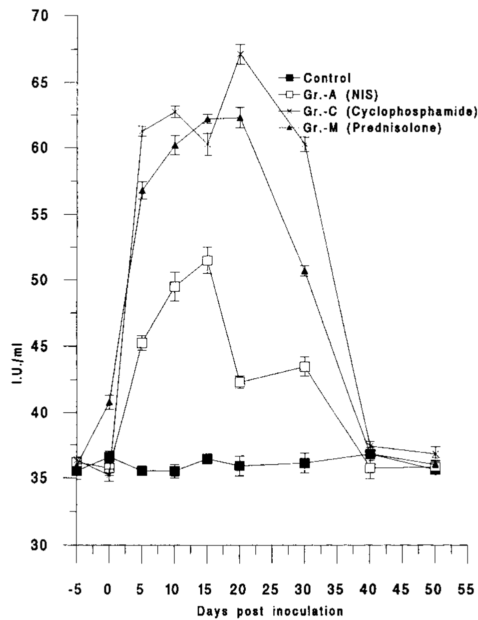

Figure 4. Changes in the aspartate aminotransferase (AST) activity in the serum of rabbits intranasally infected with *Absidia corymbifera*

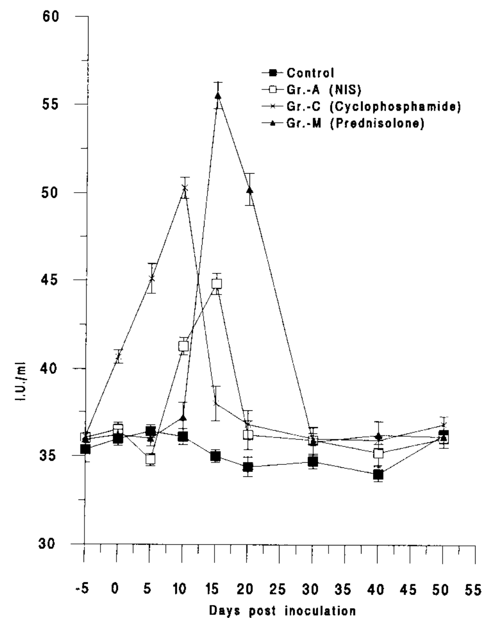

Figure 5. Changes in the alanine aminotransferase (ALT) activity in the serum of rabbits intranasally infected with *Absidia corymbifera*

Immunopathological studies

Total immunoglobulins (Figure 6) were increased significantly from 10 to 30 DPI in group A, whereas they were significantly decreased in groups C and M on days 0 and 5, followed by a significant increase at 30–50 DPI. CICs were markedly increased in group A at 15 and 20 DPI. However, in groups C and M, CICs (Figure 7) were initially significantly decreased but were significantly increased from 20 DPI.

Histopathology

The principal histopathological changes in the infected animals were mycotic lesions, confined to the lungs. These were most severe in group C and least severe in group A. At 1 DPI, there was an acute purulent inflammatory cell reaction around the fungal hyphae (Figure 8) in the pulmonary parenchyma. By 5 DPI, there were some variable-sized pyogranulomas with fungal hyphae in the centres of caseous necrosis (Figure 9), which were surrounded by neutrophils and a few macrophages, along with eosinophilic radiating clubs or eosinophilic sleeves (Splendore–Hoeppli phenomenon). Some of these granulomas were composed of macrophages, epithelioid cells, lymphocytes, plasma

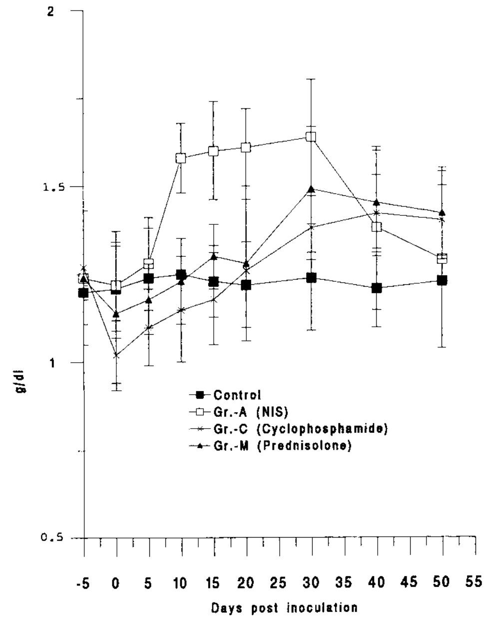

Figure 6. Changes in serum immunoglobulins in rabbits intranasally infected with *Absidia corymbifera*

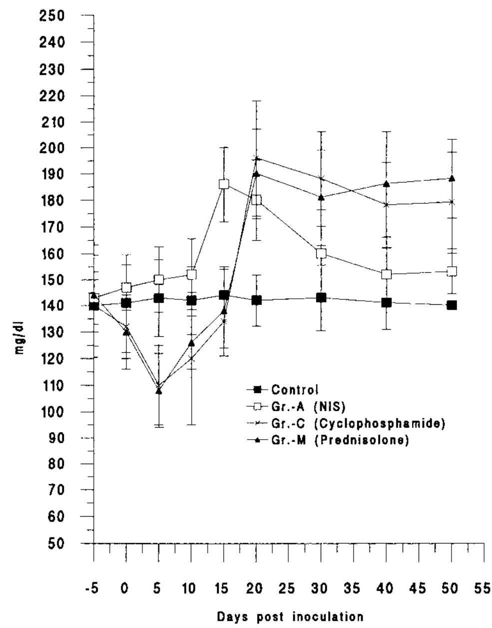

Figure 7. Changes in circulating immune complexes in the serum of rabbits intranasally infected with *Absidia corymbifera*

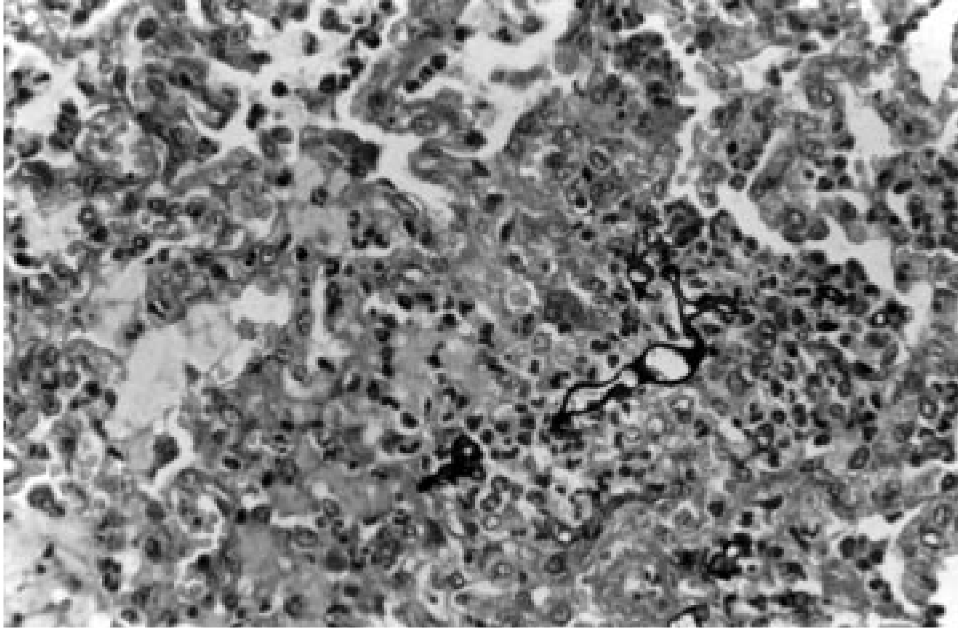

Figure 8. Lung (1 DPI). Acute purulent inflammatory cell reaction around fungal hyphae. GMS – H&E, ×300

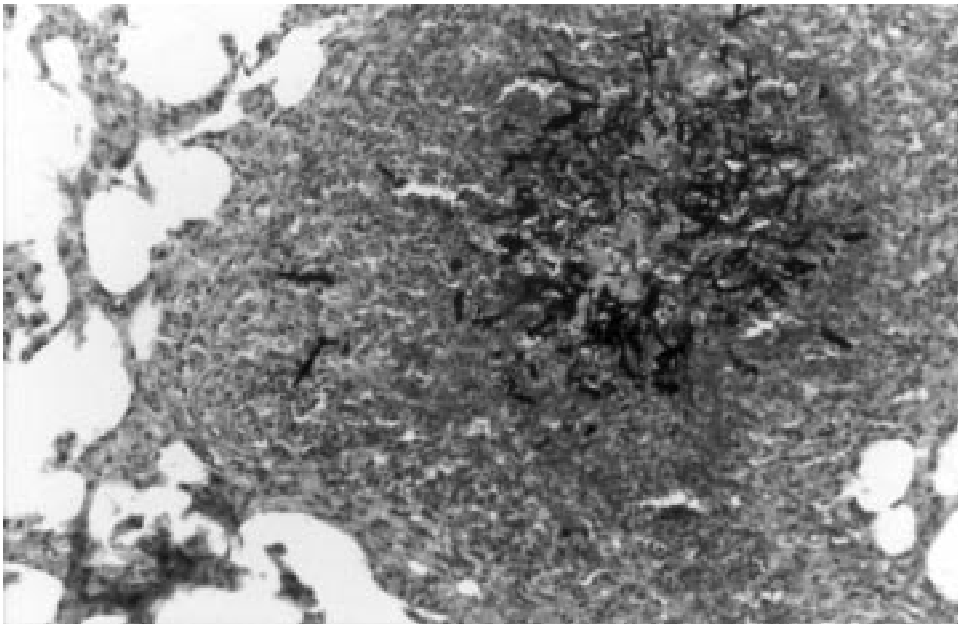

Figure 9. Lung (5 DPI). Pyogranuloma around fungal hyphae. GMS – H&E, ×150

cells and giant cells. The fungal invasion in the lungs was most severe in group C, but neutrophil infiltration around the fungal elements was most severe in group M. By 15 DPI, in group A, the distorted fungal elements were being phagocytosed by giant cells (Figure 10) and macrophages. However, variable-sized areas of caseative necrosis (Figure 11) containing fungal elements, both within and outside, were still present in the immunosuppressed groups. By 30 DPI, the lungs of group A animals showed only mild interstitial pneumonia and lymphocytic infiltration around blood vessels and bronchioles but no pyogranulomas. The lungs of the animals in groups C and M still showed a pyogranulomatous reaction with central remnants of fungal material (Figure 12). By 40 DPI, the lungs in group A were almost normal but in groups C and M there was an interstitial pneumonia, without pyogranuloma formation, and digested fungal material phagocytosed by groups of giant cells and macrophages were detected in some places. By 50 DPI, the lungs of the animals in all three infected groups were almost normal and did not show any significant pathology.

Nephrotic changes were seen in group M from 1 DPI onwards and a chronic interstitial nephritis, characterized by fibrosis, infiltration of lymphocytes in the interstitial tissue and chronic glomerulitis occurred in the animals in all three infected groups from 20 DPI onwards. There was a lymphocytic meningoencephalitis, but fungal elements could not be demonstrated in brain and kidneys by special strains.

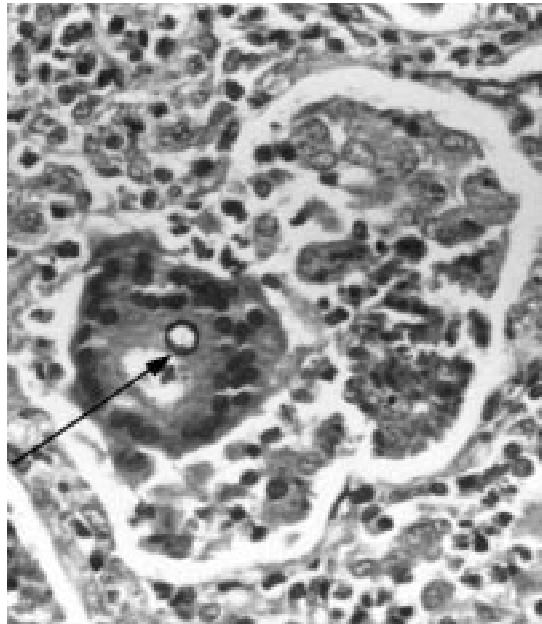

Figure 10. Lung (15 DPI). Phagocytosed fungal material (arrow) inside a multinucleated foreign body giant cell. GMS – H&E, $\times 300$

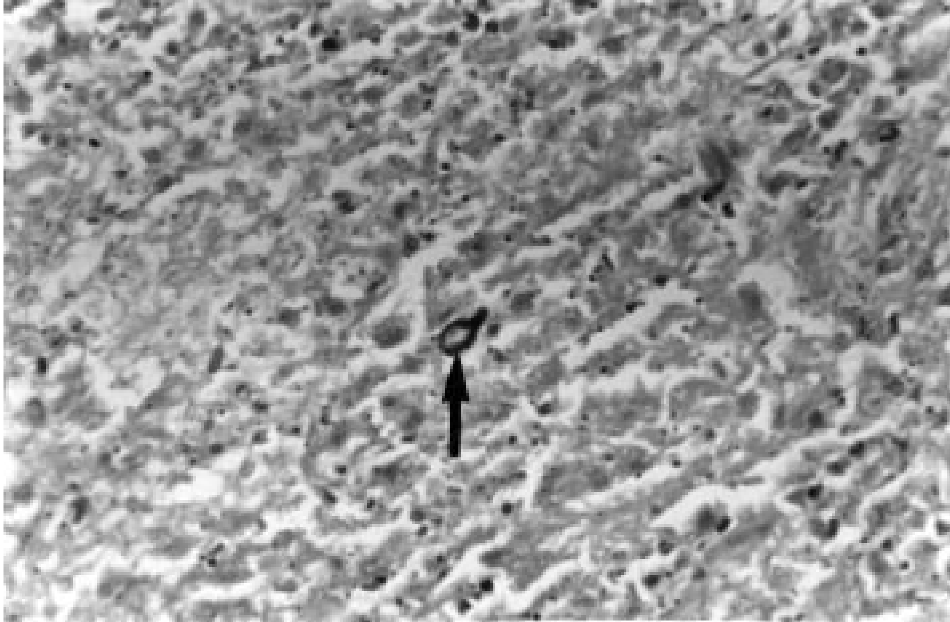

Figure 11. Lung of group C animal (15 DPI). Caseative necrosis containing a cross section of a distorted fungal hypha within it (arrow). GMS – H&E, $\times 300$

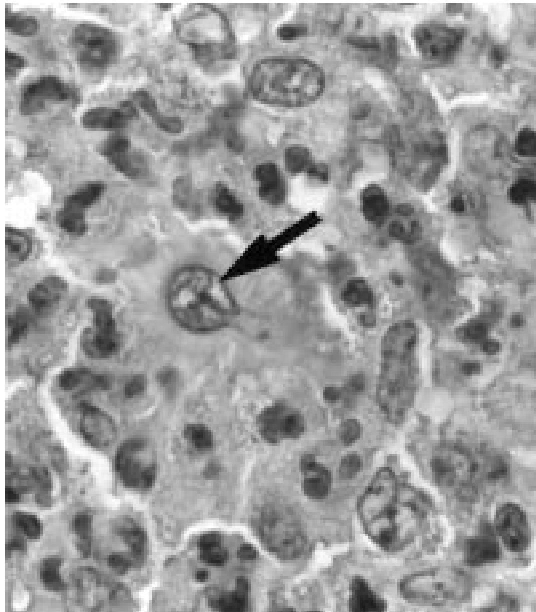

Figure 12. Lung of group C animal (30 DPI). Fungal element (arrow) in the centre of a pyogranuloma. IIP, $\times 1000$

A. corymbifera was consistently re-isolated from the lung lesions in the non-immunosuppressed group at 1–15 DPI and from those in both the immunosuppressed groups at 1–30 DPI. Fungus was not re-isolated from the brain or kidneys in any group.

There were no significant histopathological changes, nor was fungus isolated from or demonstrated histologically in any of the organs from the rabbits in the control group. *Pasteurella* was also not isolated from the lungs or blood of the experimental or control rabbits.

DISCUSSION

The clinical signs and lack of changes in Hb and PCV in our study were similar to previous findings in experimental absidiomycosis in rabbits (Corbel *et al.*, 1983; Sodhi *et al.*, 1996) and buffalo calves (Gupta *et al.*, 1999), although anaemia has been reported in clinical zygomycosis in dogs (Barsanti *et al.*, 1975) and horses (Miller and Campbell, 1983).

However, the target organ in this study appeared to be the lung, based upon our findings of confinement of mycotic lesions to the lungs as well as re-isolation of the fungus only from the lungs. This contrasts with the observations of Corbel and colleagues (1983) and Sodhi (1994), who reported that the kidneys were the main target organ in experimental absidiomycosis in rabbits. This difference was probably due to the fact that they infected their animals by the intravenous route rather than intranasally.

Leukocytosis, mainly due to neutrophilia, has been found previously in both experimental (Corbel *et al.*, 1983; Sodhi *et al.*, 1996; Gupta *et al.*, 1999) and natural (Heller *et al.*, 1971; Miller and Campbell, 1983) zygomycosis in animals. The neutrophilia may be attributed to an acute inflammatory reaction along with extensive tissue damage, particularly in the lungs (Benjamin, 1985). However, the lymphopenia and neutropenia in groups C and M appear to have been effects of the immunosuppressants. Cyclophosphamide is cytotoxic and mainly causes neutropenia (Tizard, 1987; Koptopoulos *et al.*, 1992; Plumb, 1994), while corticosteroids, such as methylprednisolone, mainly affect the lymphocytes (Coles, 1986; Jain, 1986). Moreover, the lymphopenia and the neutrophilia associated with corticosteroid administration may be brought about by redistribution of these cells in the various body compartments (Claman, 1972; Cohen, 1972; Vincent, 1977; Fauci, 1979; Jain, 1986; Guelfi *et al.*, 1985; Kaneko, 1989).

The marked increase in total serum proteins in the animals in group A must have been due to an increase in globulins (Kaneko, 1989), as the serum albumin concentration remained unaltered. A similar rise in total proteins in experimental *A. corymbifera* infections in buffalo calves was also recorded by Gupta and colleagues (1999). Conversely, the initial drop in total serum proteins in the cyclophosphamide- and methylprednisolone-treated groups may be attributed to decreased serum globulin levels (Tizard, 1987; Pedalkar *et al.*, 1991). The hypoglobulinaemia in steroid-treated animals may have been associated with the lymphopenia (Coles, 1986; Jain, 1986; Kaneko, 1989). The increased creatinine and BUN levels observed in all three infected groups and the increased BUN in group M may be due to the renal lesions demonstrated by histopathology. The steroid may also have led to increased catabolic breakdown of

proteins, resulting in increased creatinine and BUN values (Kaneko, 1989). Similar observations have been recorded by several other workers (Corbel *et al.*, 1983; Sodhi *et al.*, 1996; Gupta *et al.*, 1999) in studies on experimental absidiomycosis in animals. The increased serum AST and ALT levels in all three infected groups indicate soft-tissue damage (Kaneko, 1989), as was seen in the lungs and kidneys. Gupta and colleagues (1999) also recorded increased levels of AST and ALT in experimental absidiomycosis in buffalo calves.

The elevated total serum immunoglobulins in all three infected groups was possibly due to the increase in polyclonal antibodies seen in mycotic infections of animals and man (Howard, 1985; Rippon, 1988; Arora *et al.*, 1991). A similar rise in serum immunoglobulins was also reported by Gupta and colleagues (1999). The initial fall in total serum immunoglobulins in groups C and M might be the effect of the immunosuppression caused by cyclophosphamide (Pedalkar *et al.*, 1991) and the corticosteroid (Tizard, 1987). Chattopadhyay (1989) observed that immunosuppressed sheep and goats infected with *Aspergillus* had a poor and delayed immunological response. The significant increase in CICs also correlated with the histopathological lesions of glomerulonephritis. Slater and colleagues (1983) also reported glomerulonephritis mediated by immune complexes in pulmonary aspergillosis.

The dose of the immunosuppressant drugs used in this study was relatively low and the period of immunosuppression, 10 days around the date of infection, was relatively short. Accordingly, it is probable that the immunosuppressive effect had worn off well before the end of the study, so that the animals that had been treated with either cyclophosphamide or methylprednisolone would by then be reacting in the same way as those that had not received any immunosuppressant. This may be why their reticuloendothelial system appeared to have become active and their macrophages and giant cells had phagocytosed most of the fungal material by 40 days after infection. In AIDS and cancer patients, the immunosuppression is continuous, and secondary organisms such as opportunistic fungi can continue to proliferate.

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