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# Role of antibodies and effect of BCG vaccination in experimental candidiasis in mice

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## Abstract

The role of humoral antibodies and the effect of BCG vaccination were studied in the experimental candidiasis in mice. The antibody suppressed, B-cell deficient animals were prepared by repeated administration of rabbit anti-mouse-$\mu$-antiserum to the new born mice from birth onwards. Such immunodeficient animals along with controls were infected intravenously with *Candida albicans,* to study the course of candidal infection. It was observed that B-cell-deficient animals were found to be more susceptible to candidal infection than the controls, as indicated by their steady loss of body weight, longer mean time to death and higher viable counts of candidal cells in different organs. The anti-candidal antibodies were absent in all B-cell-deficient animals but present in the controls. These results suggest that antibodies make a contribution in protection against candidal infection in mice. The BCG vaccinated animals were prepared by repeated intravenous administration of BCG to mice and these vaccinated animals along with unvaccinated controls were challenged intravenously with *C. albicans,* to study the course of candidal infection. It was observed that BCG vaccination prolonged meantime to death and reduced the number of candidal cells in their kidneys.

## Introduction

The immune mechanisms in candidiasis are not clearly understood. The clinical evidences have established the definite importance of cell-mediated immune response (CMIR) in chronic mucocutaneous candidiasis, a unique form of the disease, often associated with T-cell defect (2, 9). However, experimental evidences suggest that CMIR may not be the major defence mechanism in systemic candidal infection in mice, as congenitally thymus-deficient (nude) mice are found to be more resistant to candidal infection than the normal controls (3, 23). The experimental data obtained in recent years indicate that host-defenses against candidal infection are dependent on neutrophils (8, 24) as well as macrophages (5, 21, 25). Reports are also available where it has been shown that a number of heterologous micro-organisms and its extracts can provide non-specific resistance to mice against candidal infection (4, 15, 29). Regarding the role of humoral antibodies in protection, the evidences are contradictory. Many investigators have failed to protect the mice against candidal infection through passive immunization (6, 7), whereas others have shown that passive transfer of immune serum is effective in protection against candidal infection in mice (18, 20).

In our study using an *in vitro* system, we have reported previously that both activated macrophages and antibodies have the potential to inhibit the growth of *C. albicans* (12). The purpose of the present study was two fold: (i) to investigate the role

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of humoral antibodies in experimental candidiasis by employing the antibody suppressed, B-cell-deficient mice and (ii) to investigate the effect of immuno-stimulation through BCG vaccination against candidal infection.

## Materials and methods

### *Animals*

Swiss albino mice of either sex weighing 20 to 25 g were obtained from Experimental Animal Facility, All India Institute of Medical Sciences, New Delhi. They were caged in groups of ten, fed on pellet diet and water given *ad libitum*.

### *Microorganisms*

A strain of *C. albicans* was obtained from the departmental stock collection. It was then passaged regularly on Sabouraud dextrose agar and the pathogenicity was tested by inoculating into the mice. The yeast cells were grown on Sabouraud dextrose agar for 24 h at 37 °C. The organisms were then harvested by centrifugation, washed twice in sterile physiological saline and suspended to the appropriate concentration in sterile saline. The viability of the yeast cells was determined to be 92 to 95% by plating the suspension onto Sabouraud dextrose agar and comparing the colony forming units (cfu) with direct counts in a haemocytometer.

The Danish strain of BCG (No. 1331) was supplied as a lyophilized preparation (BCG vaccine Laboratory, Guindy, Madras) and suspended in sterile saline before use.

A strain of *Listeria monocytogenes* obtained from the departmental stock collection was passaged regularly on brain heart infusion broth and used for the listericidal assay.

### *Anti-μ suppression*

The detail methodology for preparation of mouse IgM, rabbit anti-mouse-μ anti-serum and B-cell-deficient (μ-suppressed) mice has been described earlier (13). Briefly, to raise mouse IgM, the mice were immunized intraperitoneally with heat killed *Salmonella typhi* (non-motile strain). The mice were then bled to death and the sera were collected. The agglutination titre of the pooled serum was determined by the Widal test, before and after treatment of the serum with 2-mercaptoethanol (2-ME). It was shown that the anti-serum was 2-ME sensitive, which indicated that the antibodies were of pure IgM in nature.

Rabbits were then immunized intravenously with the agglutinated bacteria (*S. typhi*) and the whole immunization procedure was repeated to raise the high titre anti-mouse-μ antibodies. One week after the last injection, rabbits were bled by cardiac puncture and the sera were separated. The antiserum reacted with pure mouse IgM (Cappal Lab., USA) in both immunodiffusion and immunoelectrophoresis. The immunodiffusion titre was determined to be 1:32. The purity and specificity of the rabbit anti-mouse μ antibodies were tested by a immunodiffusion test against monovalent antimouse-μ antiserum.

Swiss albino mice were bred randomly in the departmental animal house. The pregnant females were kept separately in individual cages and observed twice daily for newborns. Offsprings less than 24 h old were injected i.p. with 0.05 ml of rabbit anti-mouse-μ antiserum. They were then injected with 0.05 ml i.p. every other day for 14 days, then with 0.1 ml i.p. twice in a week for 14 days and subsequently once a week for the duration of the experiments. Control mice were injected i.p. with normal rabbit serum (NRS) from birth in the same manner. At 6 weeks of age 15 mice which had been injected with NRS from birth were chosen at random and used for the characterization of the B-cell deficiency. For the assessment of the B-cell deficiency status, the following experiments were carried out: haemagglutination test for the demonstration of haemagglutinin after immunization with sheep erythrocytes, immunoelectrophoresis for the detection of serum IgM, direct immunofluorescence test for the presence of splenic B-cells and histological examination of spleen for the demonstration of germinal centers. It was shown that anti-μ treated animals were unable to make antibodies in response to sheep erythrocytes, IgM was absent in their serum, they had very few splenic B-lymphocytes and the germinal centers were lacking in the spleen. However, the B-cell deficient animals had normal T-cell function, as they rejected the skin allograft in a similar fashion to that of controls. Furthermore, these immunodeficient animals had

normal neutrophil function, as there was no difference in their phagocytic as well as candidacidal activity with that of controls, when tested *in vitro,* following the technique of Lehrer and Cline (11).

*Vaccination of mice with BCG*

Three groups of mice were vaccinated intravenously with $2.0 \times 10^7$ colony forming units of BCG in each occasion: group 3 vaccinated thrice on days 0, 7 and 21; group 2 vaccinated twice on days 0 and 7; and group 1 vaccinated only once on day 24. On day 22, 5 mice (each) from group 3 and group 2 were employed for the listericidal assay, to assess the microbicidal potential of the macrophages of BCG vaccinated animals.

*Listericidal assay*

The detailed methodology has been described earlier (12). Briefly, the macrophage monolayers were grown in Leighton tubes containing tissue culture medium with fetal calf serum, after harvesting the peritoneal exudate cells from both vaccinated and unvaccinated control animals. The monolayers were then infected with *L. monocytogenes,* allowed to proceed for phagocytosis for 30 min at 37 ° C, washed to remove extracellular bacteria and further incubated with fresh media. The cover slips were removed from the Leighton tubes at 6 and 24 h of post-infection, stained in Jenner-Giemsa and observed under microscope. It was observed that macrophages from BCG vaccinated animals inhibited the growth of *L. monocytogenes,* whereas, the macrophages from unvaccinated control animals supported the growth of intracellular bacteria and were ultimately destroyed by the bacterial overgrowth within 24 h of post-infection, which indicated that the macrophages acquired the microbicidal potential, through BCG vaccination.

*Infection of mice*

The intravenously administered $LD_{50}$ dose of this strain of *C. albicans* was determined to be $5 \times 10^3$ viable cells for mice. In order to study the course of the candidal infection, B-cell-deficient as well as control animals were intravenously infected with a standard dose of $4 \times 10^4$ viable cells. However, the challenge dose was varied to study the course of the candidal infection in BCG vaccinated animals. On the 24th day, from the initiation of vaccination, vaccinated as well as unvaccinated control animals were infected intravenously with a rigorous challenge dose of $4 \times 10^5$ viable cells to study the mortality pattern, whereas, for the enumeration of candidal cells in the kidneys on the 16th day of post-infection, animals (from both groups) were infected with a standard dose of $4 \times 10^4$ viable cells.

*Body weight changes*

To study the pattern of body weight changes in B-cell-deficient as well as control animals after candidal infection, the weight of the individual animal was taken regularly on every third day up to a 2-week period of observation.

*Mortality studies*

Twenty mice from each group (treatment and control) were selected randomly for mortality studies. The cages were regularly inspected twice a day and the deaths were recorded. Autopsy was done to ascertain the cause of death.

*Enumeration of viable C. albicans in organs*

To enumerate the viable candidal cells in kidneys and spleen, 4 to 5 mice from each group (B-cell deficient and control) were sacrificed on the 7th, 14th and 17th day of post-infection. The kidneys and spleen of each animal were removed aseptically, homogenized separately in 2.0 ml sterile saline and serial dilutions were plated in duplicate on Sabouraud dextrose agar. Colonies were counted after 24 to 48 h of incubation at 37 ° C. The viable cells were expressed as the number of organisms (log) ± standard error. The student's t-test was used to assess the significance of the results.

To determine the viable candidal cells in the kidneys of BCG vaccinated and unvaccinated animals, 5 mice from each group were sacrificed on the 16th day of post-infection and the renal colony count was done.

*Antibody estimation*

Both B-cell-deficient and control animals were

bled through retro-orbital plexus before they were sacrificed on the 7th, 14th and 17th day of post-infection and sera were collected separately. The antibodies were estimated separately by the tube agglutination test of Sweet & Kauffman (27). Antibody titre was expressed as the highest dilution of serum agglutinating the formalinized candidal cells.

## Results

### *Course of candidal infection in B-cell-deficient mice*

It was found that both B-cell-deficient and control mice showed the loss of body weight after intravenous infection with *C. albicans*. However, the loss of body weight was more pronounced and progressive in the B-cell-deficient animals in comparison with that of controls (Fig. 1).

From the pooled data of the two separate experiments it was observed that 50% mortality was achieved by the 9th day of post-infection in B-cell-deficient animals as against the 14th day post-infection in the control animal.

Equal numbers of B-cell-deficient and control animals were infected with candidal cells and at various times after infection, the number of candidal cells in kidneys and spleen was determined. The pooled data from two separate experiments are presented in Table 1. The results show that the number of candidal cells was higher in kidneys as well as spleens of the B-cell-deficient animals in comparison with that of controls. There was a significant difference ($p < 0.001$) in the viable counts in kidneys, between the two groups of animals on the 14th and 17th day of post-infection.

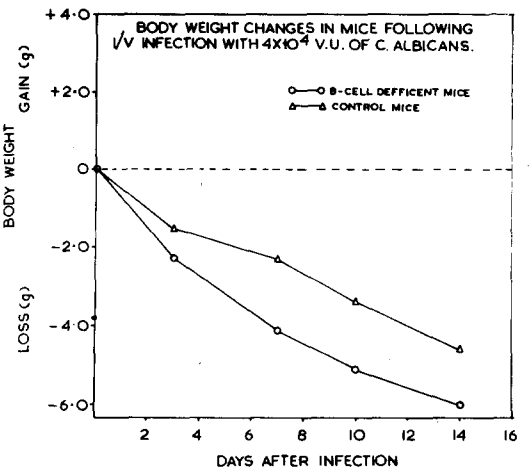


*Fig. 1.* Body weight changes in B-cell-deficient ($\mu$-suppressed) and control mice following intravenous infection with *C. albicans* ($4.0 \times 10^4$ V.U.). The values at any one point represent the mean of surviving animals in each group.

Tube agglutination test failed to detect the presence of antibody in the sera of B-cell-deficient animals infected with *C. albicans*, whereas, all the control animals showed the presence of antibody with the agglutination titre ranging from 1:32 to 1:128.

### *Course of candidal infection in BCG vaccinated mice*

In order to study the course of candidal infection in BCG vaccinated mice, the mice were first vacci-

*Table 1.* Serial viable counts in kidney and spleen of B-cell-deficient and control mice after intravenous infection with $4.0 \times 10^4$ viable units of *C. albicans*.

| Organ | Mean viable counts (log)[a] ± standard error at days after infection | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 7 | | | 14 | | | 17 | | |
| | NRS[b] | Anti-$\mu$[c] | p | NRS | Anti-$\mu$ | p | NRS | Anti-$\mu$ | p |
| Kidney | 4.81 ± 0.53 | 5.20 ± 0.13 | >0.05 | 5.55 ± 0.32 | 6.51 ± 0.21 | <0.001 | 5.81 ± 0.04 | 6.88 ± 0.19 | <0.001 |
| Spleen | 2.59 ± 0.32 | 2.78 ± 0.46 | >0.05 | 3.10 ± 0.48 | 3.51 ± 0.27 | >0.05 | 3.48 ± 0.29 | 3.79 ± 0.44 | >0.05 |

[a] Each value represents the mean of 8 to 10 animals.
[b] NRS, animals treated with normal rabbit serum.
[c] Anti-$\mu$, animals treated with rabbit anti-mouse-$\mu$ antiserum.

*Table 2.* Survival time and viable counts in kidneys of BCG vaccinated and control mice after intravenous infection with *C. albicans* on day 24 from starting day of vaccination.

| BCG vaccination given | Number of BCG vaccinations | Survival time[a] | Kidney culture[b] |
|---|---|---|---|
| | | Fifty per cent mortality achieved on day[c] | Mean viable count[d] on 16th day of post-infection |
| No | None | 6.0 | 4.56 |
| Yes | 3 (on days 0, 7 & 21) | 12.7 | 3.99 |
| | 2 (on days 0 & 7) | 11.0 | 3.84 |
| | 1 (on day 24) | 6.7 | N.D. |

[a] Animals challenged with $4.0 \times 10^5$ viable units.
[b] Animals challenged with $4.0 \times 10^4$ viable units.
[c] Each value represents the mean of 20 animals.
[d] Each value represents the mean of 8 animals.

nated with a graded dose of BCG and then challenged with *C. albicans*. It was observed from the pooled data of the two separate experiments that the 50% mortality was achieved by the 12th day of post-infection in the vaccinated animals (group 2 and 3) as against by the 6th day in the unvaccinated controls. However, the mortality rate of the animals in vaccinated group 1, was similar to that of controls.

It was found that the renal colony count was low in the vaccinated animals in comparison with that of controls. The results of the mortality study and viable count are summarised in Table 2.

## Discussion

The selective depletion of B-lymphocytes by anti-$\mu$ treatment to the new born mice has been extensively studied by a number of investigators (10, 14, 19). In our laboratory, this B-cell-deficient mice model has been employed to study the cryptococcal infection in mice (16). The effectiveness of $\mu$-suppression in the mice used in the present experiments was confirmed by the absence of IgM in the serum of all mice and the presence of very few (2 to 4%) splenic Ig-positive lymphocytes in the spleen of randomly selected B-cell-deficient ($\mu$-suppressed) mice.

The results of the present study show that the B-cell-deficient animals were found to be more susceptible to candidal infection than the controls, as evidenced by their more pronounced loss of body weight, longer mean time to death and higher colony count in their kidneys and spleen. However, none of the B-cell-deficient animals showed the presence of anticandidal antibodies, whereas, all the control animals demonstrated the presence of antibodies. Since the presence of antibodies in the control animals gave them advantage over B-cell-deficient animals, it can be suggested that antibodies contribute to the protection against candidal infection in mice.

Other workers have also reported the importance of antibodies in protection against candidal infection, Mourad & Friedman (18) have demonstrated that mice were protected against candidal infection after repeated administration of hyperimmune serum, but the mortality rate was similar to that of controls, when passive immunization was discontinued. It has been shown by Pearsall *et al.* (20) that the antibodies conferred protection to candidal infection in their mouse thigh lesion model; the thigh lesion was reduced in size when immune serum was administered repeatedly. Moser & Domer (17) have shown that the ability to form antibodies appeared to be crucial to the survival of the animals in candidal infection.

In the second part of the present study we investigated the effect of immunostimulation through BCG vaccination on experimental candidiasis in mice. The results indicate that BCG vaccination gave them advantage over the controls, as evidenced by their prolonged longer mean time to death and the reduction in the numbers of viable candidal cells in their kidneys. Several workers have also reported that heterologous microorganisms like *Listeria monocytogenes* or the extracts of

*Mycobacterium smegmatis* provided certain degree of resistance to mice against candidal infection (4, 15). In recent years a number of investigators have used BCG vaccination to increase the non-specific resistance in a variety of infections like vaccinial infection (28) and leishmanial infection in mice (26), as BCG acts through the activation of macrophages (1). So the rational for prior BCG vaccination to mice in resistance against candidal infection lies in the fact that BCG vaccination would result in the production of activated macrophages, as it has been reported that activated macrophages have greater candidacidal activity than normal macrophages (12, 25, 29). In contrast, another group of workers have reported from their renal colony count and histo-pathological observation that the activation of macrophages by BCG vaccination offered no protection against renal candidiasis, even though BCG vaccinated mice were found to be resistant to a challenge with *L. monocytogenes* (22).

We have reported earlier from our *in vitro* studies, that macrophages collected from BCG vaccinated animals show higher candidacidal activity than controls, however, this candidacidal potential is not sufficient to control the candidal infection (12). The present data also indicate that, though BCG vaccination gave advantage over the controls, it is not sufficient to protect the mice.

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