Inoculation with *Mycobacterium plhei* inhibits allergic inflammation in a rabbit model of ovalbumin (ova) sensitization*

La inoculación con *Mycobacterium phlei* inhibe la inflamación alérgica en un modelo de conejos sensibilizados con ovoalbúmina (OVA)

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La prevalencia de enfermedades atópicas y otros desórdenes autoinmunes tipo Th2 se ha incrementado en los países occidentales. Una teoría lo explica mediante la “hipótesis higiénica”, la cual plantea que una disminución en la exposición a microbios del medio ambiente conduce a afecciones atópicas. El objetivo del estudio es determinar el efecto de la inoculación con *Mycobacterium phlei* sobre la respuesta alérgica en conejos sensibilizados en estadíos tempranos post nacimiento en mucosa rectal. Treinta y dos conejos fueron divididos en 4 grupos: G1: Control normal; G2: Sensibilizado subcutáneo (SC) con ovoalbúmina (OVA); G3: Sensibilizado SC con OVA y desafiado por vía rectal con el mismo antígeno (OVA); G4: Sensibilizados SC con OVA y desafiados vía rectal con OVA previamente inoculados con *M. phlei* los días 1, 30 y 60 luego del nacimiento. Fueron utilizados los siguientes anticuerpos monoclonales: ratón anti-conejo CD4; ratón anti-conejo CD8 y ratón anti-conejo CD25 (receptor de Il-2). Las células marcadas se visualizaron mediante biotina-estreptavidina y AEC como cromógeno. **Eosinófilos:** G1: 1,2 (0,6), G2: 3,1 (0,2), G3: 13,1 (0,8), G4: 1,33 (0,4); G4 vs G3 p<0,05. **Mastocitos:** G1: 8,7 (1,3), G2: 17,6 (2,5), G3: 6,0 (1,8), G4: 7,2 (1,7); G4 vs G2 p<0,001. **CD4:** G1: 8,35 (0,7), G2: 13,4 (0,9), G3: 8,26 (0,6), G4 17,2 (1,29); G4 vs G1 and G3 p<0,05. **CD8:** G1: 6,28 (0,5), G2: 8,25 (0,8), G3: 10,77 (0,5), G4: 11,29 (1,1); **CD25:** G1: 13,2 (1,2), G2: 15,1 (1,9), G3: 20,0 (1,4), G4: 14,41 (1,9); G4 vs G3 p<0,05. En base a la evidencia presentada en este trabajo, concluimos que la inoculación temprana con *M. phlei* podría inhibir mediante modulación inmune la respuesta alérgica en conejos sensibilizados y desafiados con OVA.

**Palabras clave**

Conejo, inflamación alérgica, mucosa rectal, Mycobacterium phlei, ovalbúmina, sensibilización.

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Abstract

The prevalence of atopic disease and other TH2 autoimmune disorders have increased in Western countries. One theory explains that fact by the hygiene hypothesis, which suggests that a decrease exposure to microbes in the environment leads to atopic disorders. The aim of the present study is to determine the effect of Mycobacterium phlei inoculation on allergic response from sensitized rabbits at early stages after birth in rectal mucosa. Thirty two rabbits were divided in 4 groups: G1: Normal control; G2: Ovalbumin (OVA) sensitized subcutaneously (SC); G3: OVA sensitized SC and OVA rectal challenged and G4: OVA sensitized SC and OVA rectal challenged previously inoculated with M. phlei at days 1; 30 and 60 after birth. The following monoclonal antibodies were used: anti-rabbit CD4 for CD4+ T cells identification; anti-rabbit CD8 and anti-rabbit CD25 (IL-2 receptor marker). Botin-streptavidin system was used for detection of marked cells and AEC as chromogen. Eosinophils: G1: 1,2 (0,6), G2: 3,1 (0,2), G3: 13,1 (0,8), G4: 1,33 (0,4); G4 vs G3 p<0,05. Mast cells: G1: 8,7 (1,3), G2: 17,6 (2,5), G3: 6,0 (1,8), G4: 7,2 (1,7); G4 vs G2 p<0,001. CD4: G1: 8,35 (0,7), G2: 13,4 (0,9), G3: 8,26 (0,6), G4 17,2 (1,29); G4 vs G1 and G3 p<0,05. CD8: G1: 6,28 (0,5), G2: 8,25 (0,8), G3: 10,77 (0,5), G4: 11,29 (1,1); CD25: G1: 13,2 (1,2), G2: 15,1 (1,9), G3: 20,0 (1,4), G4: 14,41 (1,9); G4 vs G3 p<0,05. Based on the evidence presented in this work, we conclude that early inoculation with M. phlei may inhibit by immune modulation allergic response in ovalbumin sensitized and challenged rabbits.

Key words

Allergic inflammation, Mycobacterium phlei, ovalbumin, rabbit, rectal mucosa, sensitization.

Introduction

In the last decades, the prevalence of atopic disease and other TH2 autoimmune disorders have increased in Western countries1, 8, 35. One theory explains that fact by the hygiene hypothesis, which suggests an altered exposure to microbes in the environment, due to improved sanitation and personal hygiene, smaller family sizes, shorter duration of breastfeeding, immunizations and lack of serious childhood infections results in alteration of immunoregulation26, 32, 34. This hypothesis is based on an imbalance in T-helper (Th) type response and Th cell-regulatory mechanisms due to the lack of microbial stimulation16, 25, 31.

In fact, some authors describes an increased rate of allergy due to reduced exposure to environmental mycobacteria or mycobacterial products14, 36. In mice BCG vaccination leads to Th1 profile in airway allergic responses (reduce levels of immunoglobulin E, eosinophils, interleukin-4, and interleukin-5) to ovalbumin sensitization10. Inoculation of M. vaccae or BCG reduced asthmatic responses in mice15, 18. A similar pattern is observed in rats with BCG (a suppression of atopic symptoms with a reduced TH2 responses, IgE and II-4)19, 20, 21.

In a mouse model intranasal application of BCG reduced eosinophil numbers and TH2 response to ovalbumin10, 24. In some animal models it is already demonstrated that mycobacteria can restore immune homeostasis6. Mycobacteria are Gram-positive aerobic bacteria belonging to the family Mycobacteriaceae and are one of several mycolic-acid-containing genera within the order Actinomycetales17, 29. M. phlei, a commensal Mycobacterium non pathogenic for rabbit is widely distributes in the nature and has been used as adjuvant for different kinds of vaccines4.

We have previously developed a rabbit model of allergic inflammation in rectal mucosa after ovalbumin challenge in sensitized animals33. Rabbit (Oryctolagus cuniculus) is one of the animal species frequently employed as an experimental model in human and veterinary research11, 23.
The aim of the present study is to determine the effect of *M. phlei* inoculation on allergic response from sensitized rabbits at early stages after birth.

**Materials and methods**

Thirty two adult New Zealand rabbits were divided in 4 groups:

1. Group 1: (n=8) Normal control.
2. Group 2: (n=8) Ovalbumin (OVA) sensitized subcutaneously (SC).
3. Group 3: (n=8) OVA sensitized SC and OVA rectal challenged.
4. Group 4: (n=8) OVA sensitized SC and OVA rectal challenged previously inoculated with *M. phlei* at days 1, 30 and 60 after birth. *M. plhei* inoculation was developed by subcutaneous administration of 3x10⁷ bacilli per rabbit (final volume 0.1ml).

As we described in previous works, rabbits from group 2, 3 and 4 were twice subcutaneously sensitized with 70 µg OVA (Grade V Sigma) in 30 mg ALUM/ml (aluminium hydroxide)²,³. An interval of 15 days among sensitizations was developed. After 15 days post sensitization, rabbits were 24 hours fasten and G3 and G4 animals were rectal challenged with 50 mg OVA in 5 ml of Phosphate Buffered Saline (PBS).

Animals were sacrificed with sulfuric ether 4 hours after challenged, according to considerations of Ethical Comettee of Rosario School of Medicine and samples from rectum were obtained from all groups. Material for immunohistochemistry were snap frozen in liquid nitrogen with OCT protector and stored at −20 °C and cut at 8 micrometers. Samples for histology were paraffin embedded and cut at 6 micrometers.

The following monoclonal antibodies were used:

1. Mouse anti-rabbit CD4 (KEN-4, BALB/c IgG 2a, AbD SEROTEC, UK)²².
2. Mouse anti-rabbit CD8 (12.C7, BALB/c IgG1, AbD SEROTEC, UK)⁷.
3. Mouse anti-rabbit CD25 ((Kei-α1BALB/c IgG2b, AbD SEROTEC, UK)²².

Botin-streptavidin system (Sero tec Ltd. Oxford, UK) was used for detection of marked cells and AEC as chromogen. Frozen samples were fixed in cold acetone for 10 minutes and then incubate for 60 minutes at 37 °C with monoclonal antibodies. Cromotope II technique was employed to detection of eosinophils and Alcian blue pH < 1 was used for mast-cells in rectum.

Positive cells were analyzed in 200 high power fields in each group and expressed as cell mean per microscopical field by morphometric analysis⁹. Results were statistical analyzed.

Specific anti-OVA IgE titres were evaluated by passive cutaneous anaphylaxis PCA⁵.

The results were expressed as mean. The comparison between variables in different experimental groups was made by analysis of variance by ranks of Kruskal-Wallis or Mann-Whitney U test, using the statistical program Medcalc 3.6.

Samples were considered different when they had a significance level <0.05.

**Results**

Specific anti-OVA-IgE levels were evaluated by positive passive cutaneous anaphylaxis test (PCA) possitive at 160 fold dilutions in sensitized groups (G2, G3, G4). Histology of samples showed patchy mucosal edema, lymphangiectasia and eosinophil infiltration in the experimental OVA sensitized and challenged groups (G3 and G4). Eosinophils were spread on the mucosa.
underlying the epithelium. There were no visualized histopathological modifications in the control (G1) and the sensitized and unchallenged group (G2). Different cell population quantification were summarized in table 1.

**Table 1.** Cell populations in rectal mucosa from normal rabbits, ova sensitized rabbits and sensitized and locally challenged rabbits. Positive cells per high power field arithmetic mean and SE.

<table>
<thead>
<tr>
<th>Markers</th>
<th>G1: Control N = 200</th>
<th>G2: sensitized n = 200</th>
<th>G3: sensitized and challenged n = 200</th>
<th>G4: sensitized and challenged, treated with M. phlei n = 200</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>8,35 ± 0,7</td>
<td>13,4 ± 0,9</td>
<td>8,26 ± 0,6</td>
<td>17,26 ± 1,2*</td>
</tr>
<tr>
<td>CD8</td>
<td>6,28 ± 0,5</td>
<td>8,25 ± 0,8</td>
<td>10,77 ± 0,5</td>
<td>11,29 ± 1,1</td>
</tr>
<tr>
<td>CD25</td>
<td>13,2 ± 1,2</td>
<td>15,1 ± 1,9</td>
<td>20 ± 1,4</td>
<td>14,41 ± 1,9*</td>
</tr>
<tr>
<td>Mast cells</td>
<td>8,7 ± 1,3</td>
<td>17,6 ± 2,5</td>
<td>6,0 ± 1,8</td>
<td>7,2 ± 1,7**</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>1,2 ± 0,6</td>
<td>3,1 ± 0,2</td>
<td>13,1 ± 0,8</td>
<td>13,3 ± 0,4**</td>
</tr>
</tbody>
</table>

* Different from G2 and G3 p<0,05. ** Different from G2 (p<0,001). * Different from G3 (p<0,05). ** Different from G3 (p<0,05)

CD4, CD8 and CD25 positive cells from different groups were visualized in figures 1, 2, 3 and 4.

**Figure 1.** CD4 positive cells in the rectal mucosa. Sensitized and challenged rabbits. Observe the nucleus lack of staining. x 400. (Group 2).

**Figure 2.** CD 4 positive cells adjacent to the bottom of crypts from G3, sensitized and challenged rabbitss. x 100.

**Figure 3.** CD8 cells from group 4. X 100.

**Figure 4.** CD25 positive cells in the rectal mucosa from sensitized and challenged rabbits. x 400. (Group 3).

Ovalbumin is a soluble antigen which, when subcutaneously administered, induces sensitization that elicits specific IgE antibodies. Challenge with OVA in sensitized individuals produces an anaphylactic allergic response in the digestive tract. In previous works we found modifications in number of enteroendocrine cells, mast cells and eosinophils in rectal mucosa from OVA-sensitized and challenged rabbits. These changes associated to high levels of specific anti-OVA IgE, indicated an immediate hypersensitivity reaction.

In the present work we observed eosinophil infiltration, vascular congestion and lymphangiectasia...
in group 2 and 3 (sensitized and challenged). Important strengths of our study are the objective histopathological findings of allergic inflammation in rectal mucosa in sensitized animals.

Such signs become apparent 4 hours after OVA challenge. In reference to *M. Phlei* inoculated rabbits, our study demonstrated an inhibition of allergic inflammation in rectal mucosa vs group 2 and 3.

As seen in results referred to reduced eosinophil infiltration and mast cell degranulation in rabbits inoculated with *M. phlei* we conclude that early contact with mycobacteria inhibits in some way allergic infiltration and inflammation. These results are similar to that found by different authors at airways in mice.

We demonstrated a statistic significant increase in number of CD4+ cellular populations in animals from G2 and G4 as compared to control. Sensitization and oral challenge induce certain immune cells populations recruitment and migration phenomena with a decrease in naive lymphocytes. Conversely the immune activation of T cells is evidenced by an enhance of CD25 (interleukin-2 receptor) membrane receptor in sensitized and challenged rabbits (G3), these increase is strongly inhibited in *M. Phlei* inoculated animals (G4). These data is similar to that found by Saavedra *et al.*, showing evidence that mycobacterial cell-surface glycolipid are able to reduce antigen-induced proliferation of human CD4+ and CD8+ T-cell subsets associated with a decrease in cells expressing the T-cell surface activation markers CD25 and CD69.

Different mechanisms could explain these findings. Exposition to mycobacteria at early stages of life may downregulate allergic response through the induction of T regulatory cells and production of anti-inflammatory cytokines. Some authors have demonstrated that simultaneous mycobacteria inoculation and allergen sensitization may stimulate a Th1 profile able to suppress Th2 activity significantly.

In our model stimulation of T cells with OVA by subcutaneous sensitization only induced Th2-dominant responses, whereas concomitant stimulation with both OVA and *M. plhei* could caused induction of antigen-specific Th1 responses, with secretion of IFN-g, mediated by IL-12 as described by C. Obihara.

Based on the evidence presented in this work, we conclude that *M. phlei* (or *M. phlei* products) might be able to protect against the development of allergic inflammation and atopic disease. Although the exact mechanisms involved in the protective effect are not yet known, immune mechanisms involved in the defense against *Mycobacteria* and immune modulation could be reasons for observed effects on this animal model.

**References**


