Oral Immunization With *Acanthamoeba* Mannose-Binding Protein Ameliorates Amoebic Keratitis

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**Running Title:** MBP-based protection against amoebic keratitis

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ABSTRACT

Acanthamoeba mannose binding protein (MBP) mediates adhesion of the amoebae to corneal epithelial cells, a key first step in the pathogenesis of Acanthamoeba keratitis (AK), a devastating corneal infection. In the present study, we demonstrate that oral immunization with rMBP ameliorates AK in a hamster animal model and that this protection is associated with the presence of elevated levels of anti-MBP IgA in the tear fluid of the immunized animals.
**Acanthamoeba keratitis (AK)** is a rare but painful and devastating infection of the cornea that is difficult to diagnose and treat (2, 4, 8, 12, 13, 16). We have recently shown that *Acanthamoeba* express a mannose-binding protein (MBP) that mediates the adhesion of the amobae to corneal epithelial cells (3, 5, 6, 18) and that anti-MBP IgA antibodies are present in the human tear fluid of healthy individuals (G.N. Alberti, M. Garate, Z. Cao, D. Zoukhri, M. Goldstein, K. H. Wu, and N. Panjwani. Abstr. ARVO Annual Meeting, abstr. 4969, 2004). Because MBP mediates adhesion of the *Acanthamoeba* to host cells, the presence of anti-MBP specific IgA in tear fluid may provide protection against the infection by blocking the adhesion of amoebae to the corneal surface. In this respect, it has been reported that the levels of anti-*Acanthamoeba* IgA antibodies are reduced in tears (1) and sera (17) of patients with AK. The goal of the present study was to test the hypothesis that the mucosal immune system resulting in the production of anti-MBP IgA may be instrumental in providing protection against AK.

Chinese hamsters (Cricetulus griseus, 4-6 weeks old, Cytogen Research and Development, Inc. West Roxbury, MA) were orally immunized with a highly purified preparation of recombinant MBP (rMBP) (5) according to the regimen depicted in Figure 1. All animals were handled in accordance with recommendations of the NIH Guide for the Care and Use of Laboratory Animals. Prior to immunization, corneas of all animals were examined under an operating microscope to exclude animals with any preexisting corneal disease (11). Two trials, MBP trials 1 and 2, were conducted. In trial 1, a group of 6 animals was immunized by oral administration of 100 µg of rMBP plus 10 µg of neutralized cholera toxin (CT) in 100 µl of PBS. The animals were boosted by administration of three consecutive weekly doses of the same amount of antigen and CT.
A control group of 6 animals was sham-immunized with vehicle (PBS) alone. We have previously shown that the control animals immunized with CT alone as well as control antigens (e.g. lysozyme), are not protected against AK in this model (9). In MBP trial 2, the same immunization regimen was used except that the animals were immunized with 200 µg of rMBP instead of 100 µg rMBP used in the MBP trial 1. Seven days after the fourth oral immunization, the infection was induced by placing *A. castellanii* (ATCC No. 30868, axenically cultured, >95% trophozoites)-laden contact lenses on scarified corneas of one eye of each animal as described previously (7, 9). After 4 days, corneas were examined under a dissecting microscope to assess the severity of infection based on the presence of corneal infiltration, neovascularization, and corneal ulceration. On the scale of 0-4, zero represented no infection, and scores 1 to 4 indicated that 10%, >10 - <25%, 25 - <50% and > 50% of the cornea, respectively, was affected by the disease. A score of 1.0 on any of the criteria represented infection. Clinical-severity scores were analyzed by the Mann-Whitney test. In this model, the AK has a self-limiting course of 3 weeks (7, 9).

As expected (7, 9), control animals immunized with vehicle alone developed severe keratitis (Mean severity score on postinfection day 5: 1.85 ± 0.5 [N=6, trial 1]; 1.83 ± 0.5 [N=5, trial 2]) that was cleared by day 22 (Figure 2A). In MBP trial 1, all 6 animals were protected (Figure 2A). In MBP trial 2, 4/6 animals were protected (not shown; severity score: postinfection day 5: 0 [2 animals], 0.8 and 1.0; day 7: 0 [2 animals], 0.12 and 0.63), and two were not protected (severity score: postinfection day 5: 2.0 and 2.25; day 7: 1.25 and 1.62). On average, oral immunization with MBP in trials 1 and 2 reduced the severity of infection by 86% and 57%, respectively, and reduced the duration of the disease by 12 and 5 days, respectively. Overall, significant protection was
achieved in both trials, but the lower dose (100 µg) appeared to be more efficacious. Additional studies are needed to optimize the dose and immunization regimen for achieving maximum protection.

In an effort to understand the mechanism by which the oral immunization provides protection against AK, we measured the anti-MBP IgA in tears of immunized animals using an ELISA procedure described previously (7, 9). There was no significant difference in the anti-MBP IgA levels between the control and the immunized groups in the tear samples collected on day 0 and day 7 (not shown). In contrast, anti-MBP IgA levels were significantly higher in the immunized animals compared to the control group in the tear samples collected on day 14 (Figure 3A) as well as day 21 (Figure 3B). It is noteworthy that 2/6 animals that were not protected in the MBP trial 2 had antibody titers that were similar to the animals which were fully protected (Average O.D. values of ELISA assays on day 21 at protein concentration 75 µg/ml: [i] control animals: 0.016±0.003 [N=6]; [ii] immunized animals: a. protected: 0.047±0.002 [4/6]; b. not protected: 0.045[2/6]). This may suggest that immune response against a specific region of MBP may be required to provide protection, and that the animals which were not protected, may have elicited immune response against a distinct region(s) of MBP compared to the animals which were protected. This reasoning stems from the observations of Petri (14, 15) and Lotter (10) demonstrating that in a gerbil model of Entamoeba infection, immune response against specifically the C-terminal domain encompassing the carbohydrate recognition domain (CRD) of E. histolytica galactose-specific lectin is protective whereas the immune response against the N-terminal domain of the lectin is not protective, and, in fact, it exacerbates the disease.
We have recently cloned and sequenced *Acanthamoeba* MBP (5) and have shown that the architecture of the lectin is characteristic of a cell surface receptor consisting of a large extracellular domain, a single pass transmembrane domain, and a short cytoplasmic domain which is located at the C-terminus. At present, the exact location of the CRD in the amoeba MBP is not known. The study described in this manuscript lays the foundation for future studies to pinpoint the exact location of the CRD and to characterize individual domains of the amoeba MBP with respect to their role in host-parasite interactions.

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**REFERENCES**


**FIGURE LEGENDS**

**Figure 1:** Immunization regimen and induction of AK. Hamsters were orally immunized by four weekly doses of rMBP, tears were collected on days 0, 7, 14 and 21, and the infection was induced on day 28 by placing amoebae-laden contact lenses on the surface of the cornea. The contact lens was removed from each eye on day 4 postinfection, and the severity of the infection was scored either daily or every other day from postinfection days 5-22.

**Figure 2:** Immunization of Chinese Hamsters with rMBP ameliorates amoebic keratitis. **A:** Hamsters were orally immunized with rMBP (100 µg) and were challenged with *A. castellanii*-infected contact lenses. Severity of infection was recorded beginning postinfection day 5. Severity score was significantly lower for the rMBP immunized group (solid circles) compared to the sham immunized control group (open circles). Data are presented as mean ± SE (N=6 for each group). **B:** Representative photographs of eyes on postinfection day 12. *p<0.05 compared to control group.

**Figure 3:** Tears of rMBP immunized animals contain elevated levels of anti-MBP IgA antibodies. Tears were collected during the immunization period on days 0, 7, 14 and 21 and were analyzed individually for the presence of anti-MBP IgA by ELISA. The assay
was performed using various dilutions of tears from 9.4 - 75 µg/ml protein concentration. Results of tears collected on day 14 (A) and day 21 (B) are shown. Data are presented as mean ± SE (N=6 for each group). *p<0.05 compared to control group. No difference in the antibody level was detected between the immunized and control groups in the tear samples collected on day 0 and day 7 (not shown).
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Figure 1. Garate et al.

- Primary Immunization
- First Booster 1 week
- Second Booster 1 week
- Third Booster 1 week
- Keratitis Induction
- Contact Lens Removal Day 4
- Days 5-22
- Collection of Tears
- Recording of Severity Score
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Figure 2. Garate et al.
Figure 3. Garate et al.

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A

B

Figure A and B show the concentration of MBP and rMBP compared to vehicle control at different concentrations. The graphs indicate a significant increase in OD 450 nm with increasing protein concentration for both MBP and rMBP compared to the vehicle control.

* denotes significant difference from the vehicle control.