Role of Adaptive Immunity in the Pathogenesis of Candida albicans Keratitis

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PURPOSE. Innate immunity had been thought to be critical in the pathogenesis and prognosis of fungal keratitis. This study was conducted to determine whether experimental Candida albicans keratitis (CaK) induces an adaptive immune response.

METHODS. Experimental murine CaK was induced by intrastromal injection of C. albicans spores, and fungal pneumonia was induced by intranasal inhalation of spores. Active immunization was accomplished by subcutaneous injection of heat-inactivated spores. Serum was collected at different times after the induction of primary or secondary CaK for the measurement of IgA, IgG, IL-4, and IFN-γ. Immunohistochemistry was used to detect immunoglobulin deposition and lymphocyte infiltration in diseased corneas.

RESULTS. After intrastromal injection of C. albicans spores in immunocompetent mice, typical CaK occurred, and the corneas healed in 3 weeks. When recovered corneas were challenged again with spores, they developed milder CaK and healed faster than with primary CaK. Mice that had recovered from pulmonary infection or had been immunized also showed increased resistance to CaK. Compared with naive mice, the mice that had previously encountered C. albicans produced more IgG and IgA in serum and more immunoglobulin deposition and lymphocyte infiltration in corneas on secondary CaK induction. Cytokines assays showed that the immune response induced by CaK was biased toward the T-helper (Th)1 type.

CONCLUSIONS. Th1-type adaptive immune response and immunologic memory were induced by C. albicans keratitis, and previous contact with Candida preparation enhanced the resistance of the host to subsequent corneal challenge with the same fungus. Active immunization might be an effective strategy to prevent fungal keratitis in populations at high risk. (Invest Ophthalmol Vis Sci. 2009;50:2653–2659) DOI: 10.1167/iovs.08-3104

Fungal keratitis (FK) is among the most dangerous ocular infections and can threaten eyesight if not properly controlled. The proportion of corneal infections caused by FK varies throughout the world. In New York in the 1980s, for example, approximately 1% of all observed infectious corneal diseases were designated FK, whereas this number was 35% in Florida during that same period. The best known predisposing factors include trauma in underdeveloped countries or contact lens wear in developed countries; hot and humid weather is a further risk factor in all countries. Because of the specific structure of the cornea (thin and transparent) and the growth properties of fungi, FK manifests a fast and vicious progress and often leads to total sight loss in days. Better understanding of the pathogenesis of FK is necessary for the development of more effective therapeutics or intervening surgery. Unfortunately, progress in this field has been slow, and many issues remain unclear. For example, the molecular mechanisms underlying the interaction between pathogens and corneal components remain to be revealed, though it is agreed that filamentation of fungus mediated by molecules such as transcription factor or protease is critical for initiating the process. The host reaction to corneal fungal invasion is also unclear. Large numbers of polymorphonuclear cells infiltrate the infected corneas, suggesting that innate immunity involving various Toll-like receptors (TLRs) is the dominant host response to FK. We believe, however, that antigen-specific adaptive immunity is also involved in fighting the invading fungus. Our hypothesis is based mainly on existing data that abundant professional antigen-presenting cells, such as dendritic cells and macrophages, reside in the resting corneas and readily mature and migrate to draining lymph nodes on stimulation. Adaptive immunity had been shown to be involved, as either a protective or a destructive factor, in the pathogenesis of keratitis caused by bacteria, virus, or acanthamoeba, clearly demonstrating the capability of the cornea to start an adaptive immune response against pathogens. Wu et al. observed that cyclophosphamide, a potent immunosuppressive drug that works primarily by inhibiting lymphocyte proliferation, increased the severity of FK, implying that lymphocytes might be involved in the pathogenesis of FK. In this study we confirm the hypothesis by showing that fungal infection of the cornea induces an adaptive immune response and an immunologic memory in host animals. Furthermore, we show for the first time that priming the murine immune systems by infecting other organs with live spores or by vaccination with inactivated spores helps to resist corneal infection by the same fungus.

MATERIALS AND METHODS

Candida albicans Spore Preparation and Lysate

Candida albicans, strain MYA-2876 (ATCC, Manassas, VA), was cultured routinely in accordance with the Shandong Eye Institution Biosafety Code. Spores were harvested, washed, and suspended in saline buffer at a concentration of 5 × 107/mL to make the C. albicans spore preparation (CASP). To prepare CASP lysate for in vitro stimulation of splenic cells, CASP was heated to 60°C for 1 hour; this was followed by three freezing and thawing cycles. The preparations were sonicated on ice for 20 minutes at 18,000 Hz, and the lysate was centrifuged at 1200g for 30 minutes. The supernatant was referred to as C. albicans spore-soluble total antigen (CAS-STA) and was used as described here.

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C. albicans Keratitis Model

Balb/c mice were purchased from the Beijing Pharmacology Institute, Chinese Academy of Medical Sciences (Beijing, China). The ARVO Statement for the Use of Animals in Ophthalmic and Vision Research was observed throughout the study. All mice, of both sexes, were 6 to 8 weeks old when recruited into the experiments. For induction of fungal keratitis, the intrastromal injection method was used under anesthesia, with minor modifications. Briefly, the corneas were pierced near the center with a 30-gauge needle to the depth of the stroma. Then a 33-gauge needle with a 30° bevel (Hamilton, Reno, NV) was used to inject 1 µL CASP into the center of the cornea. In the sham-infection group, the same volume of saline buffer was substituted for the fungal suspension. Corneas were monitored daily with a slit lamp equipped with a digital camera. A 12-point scoring system, based on the fungal suspension. Corneas were monitored daily with a slit lamp equipped with a digital camera. A 12-point scoring system,13 based on the intensity and extent of the corneal opacity, was adopted to evaluate the development of the disease.

Experimental Schedule

In total, three priming protocols were used in this study (Fig. 1): pre-fungal keratitis (pre-FK), pre-pulmonary infection (pre-PI), and pre-active immunization (pre-AI). For pre-FK groups, mice were challenged with live CASP to allow CaK to develop and resolve without any medical or surgical manipulation. Age-matched naive mice injected with the same volume of saline buffer were used as a sham-infection control to monitor the effect of the injection process alone. In the pre-PI protocol, the mice were treated for 3 consecutive days with intraperitoneal injections of methylprednisolone (100 mg/kg body weight). On days 4, 5, and 6, the mice were anesthetized and held upright with the nasal cavity upward. CASP (15 µL of a 106/mL suspension) was slowly administered to each side of the nasal cavity in turn, and the mice remained in position for 3 minutes. This dose of microbes was predetermined to be the optimal dose for reliably producing effective pulmonary infections without causing death. Significant loss of body weight was used as an index of successful induction of pneumonia. In the pre-AI groups, CASP was inactivated and mixed with complete Freund adjuvant (Sigma, St. Louis, MO) for subcutaneous immunization. The first dose consisted of 4 × 104 heat-inactivated spores injected into four to six spots along the flank. Booster injections with 104 heat-inactivated spores were repeated 2, 4, and 6 weeks later, respectively. All the primed mice and age-matched naive controls were left untreated until 8 weeks after the first priming. Then intrastromal rechallenge with CASP was performed to induce CaK in all mice. Starting with priming and continuing throughout infection, serum was harvested by tail bleeding and mixing with an equal volume of EDTA as an anticoagulant. In addition, at certain time points, several of the mice from various groups were killed, and splenic cell cultures were set up to detect their response to in vitro CASP challenge. The numbers of mice and samples used are indicated in the Results.

Pre-FK &
Pre-PI * * * $ $ $ &
Pre-AI # # # # &
Control &
-8W (1 2 3 4 5 6 7) -6W -5W -4W -3W -2W -1W 1W 2W 3W 4W 5W 6W 7W
d0 d7 d14 d21
Sampling S S S S S S
Splenic cell culture C C C

FIGURE 1. General timeline of the experiments. (&) Intrastromal injection of 5 × 104 live spores. (1) Pretreatment with methylprednisolone. ($) Intranasal administration of live spores (5 × 105). (2) Subcutaneous injection of 4 × 105 heat-inactivated spores mixed with CFA. S, serum sampling; C, splenic cell culture.

Splenic Culture in Response to CAS-STA

Mice were killed, spleens were removed, and single-cell suspensions were prepared by pressing the spleens through 100-µm meshes and lysing erythrocytes with Tris-NH4Cl. Cells were cultured at 2 × 107/mL in 24-well plates in the presence or absence of CAS-STA (corresponding to 1 × 106 spores/mL) for 48 hours. At the end of this time period, part of the supernatant (500 µL) was harvested for cytokine detection, and the cells in the wells were assayed according to a colorimetric method (Cell Counting Kit-8; Dojindo, Kumamoto, Japan).

ELISA for Cytokines

IFN-γ and IL-4 were detected side by side in serum or culture supernatant with the use of mouse IFN-γ and IL-4 ELISA-based immunosassay systems (OptEIA Sets; BD PharMingen, San Diego, CA), respectively, according to the manufacturer’s protocol. Standard curves were run at the same time and were used to calculate the concentration of cytokines in the samples.

ELISA for Immunoglobulins

Pathogen-specific immunoglobulins were detected according to a protocol adapted from Wozniak et al. Briefly, heat-inactivated CASP was diluted in coating buffer (0.1 M sodium carbonate, pH 9.5) to a final concentration corresponding to 106 spores/mL and then was added to ELISA plates (Nunc, Naperville, IL) at 100 µL/well. After coating overnight at 4°C, the plates were blocked for 2 hours at room temperature with 10% bovine serum in PBS. Mouse serum and splenic cell culture supernatant samples were diluted and added to the ELISA plates, and the incubation continued for 2 hours at room temperature. After two washes with PBS-Tween wash buffer, HRP-conjugated goat anti-mouse IgM, IgA, or IgG (Southern Biotechnology, Birmingham, AL) was added and incubated for 1 hour. After seven washes, tetramethyl benzidine (TMB) substrate (BD PharMingen) was added and developed for 30 minutes before 1 M H2PO4 was added to stop the reaction. The plates were read at 450 nm and 570 nm, respectively, and the Δ (OD450 – OD570) were obtained for each well.

Immunohistochemistry for IgA/G and CD4/8 Lymphocytes

Corneas removed from various groups of mice at day 7 were embedded in OCT (Sakura Finetek USA., Inc., Torrance, CA), cryosectioned, and fixed with carbon tetrachloride (for IgA and IgG detection) or acetone (for CD4 and CD8 detection). FITC-conjugated anti-mouse IgG and TRITC-anti-mouse IgA (Southern Biotechnology), or FITC-anti-mouse CD8 plus PE-anti-mouse CD4 (BioLegend, San Diego, CA), were mixed to 5 µg/mL each, and the sections were stained for 30 minutes at 37°C, followed by three washes with PBS-T. The sections were observed under a fluorescence microscope (E800; Nikon, Tokyo, Japan).

RESULTS

C. albicans Keratitis Progression in Immunocompetent Mice

Few reports are available on the long-term outcome of FK (caused by any fungal species) in mouse models. In this study, we found that a relatively low dose (5 × 103) of spores induced obvious CaK in all mice when the intrastromal challenge model was used and that the highest disease symptom score appeared between day 7 and day 10. After this time point, the mice began to recover even without therapeutic treatment, and virtually all the corneas returned to their normal transparent state by day 21 (Fig. 2). Injection of saline buffer (sham infection) also caused mild opacity in the early after infection, but this abnormality disappeared quickly, usually within 1 week. Approximately 5 weeks after the complete disappearance of the primary CaK, the corneas that recovered from infection...
were again challenged with CASP. Significantly, they developed less severe disease than in response to the previous infection, and the corneas regained transparency more quickly than before (Fig. 3). This indicated that corneal infection with *C. albicans* endowed the animal with stronger resistance to the same fungus. In addition, pilot studies and parallel controls showed that male and female mice responded equally to CaK challenge (Fig. 4B).

**Alleviated Infection Disease in Primed Mice**

Next, we looked into whether infection of other organs with live fungi or immunization with heat-killed fungi was effective as a vaccination for CaK. We used the model of *C. albicans*-induced pneumonia, a common infection in various parts of the world. By optimizing the number of spores used, the mice could recover from successfully induced pneumonia that was confirmed by histology (data not shown). Indicators of a successful lung infection and recovery included changes of fur texture and body weight. When recovered mice were subjected to the induction of CaK, they manifested milder disease and quicker healing than did age-controlled mice undergoing primary CaK infection (Fig. 4). The pre-AI protocol with heat-inactivated spores produced a similar but stronger vaccination effect, demonstrated by lower disease scoring and quicker recovery of the infected corneas than occurred in control and pre-PI groups (Fig. 4).

**Memory-type Immune Response to Secondary Corneal Infection**

We then studied the pattern of the immune response to CaK in mice that encountered fungi in the cornea for the first or second time. After intrastromal injection of CASP into naive mice, we observed that class-switched IgG and IgA antibodies, in addition to IgM antibodies, were induced (Fig. 5). When the recovered mice were challenged with the induction of secondary CaK, higher levels of IgG and, to a lesser extent, IgA, were detected in the sera of the mice (Fig. 5). Mice of pre-PI and pre-AI groups also produced more IgG than age-controlled naive mice after CaK induction (Fig. 5). When the splenocytes were harvested at different times after the start of secondary CaK and tested for immunoglobulin production in vitro, the cells from pre-FK, pre-PI, and pre-AI mice produced more antibodies, with or without in vitro stimulation by CAS-STA, compared with those from age-matched control groups (Fig. 5). Similarly, the splenocytes of pretreated mice manifested stronger proliferation response than those from age-matched control groups (Fig. 6).

**Th1-Type Cytokine Production Induced by CaK**

IFN-γ and IL-4 were examined as representatives of Th1-type and Th2-type cytokines, respectively. In mice with primary CaK and mice with secondary CaK, IFN-γ levels started to increase earlier than those of IL-4. Additionally, IFN-γ/IL-4 ra-

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932960/) **Figure 2.** Progression of disease during primary *C. albicans* keratitis. (A) Slit lamp pictures of an infected cornea at different times. (B) Scoring of all *Candida*-infected corneas and sham-infected corneas.

![Figure 3](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932960/) **Figure 3.** Corneal dynamics during primary and secondary fungal keratitis. (A) Representative cornea at different times. (B) Scoring of corneas after primary or secondary challenge of CaK. *n* = number of mice in that group at the specific time points.
FIGURE 4. Effect of priming on subsequent fungal keratitis expression. (A) Typical disease expression of fungal keratitis induced in mice that recovered from pulmonary infection (pre-PI) or active immunization (pre-AI) compared with controls. (B) Scoring of disease in all groups, including the pre-FK for comparison.

FIGURE 5. Immunoglobulin levels in mouse serum or splenic cell culture supernatant. Serum samples were collected at different times from mice that developed primary CaK or sham infection (left) or from mice that had been primed and developed secondary CaK (middle). In some experiments, the splenocytes were cultured in vitro and stimulated with CAS-STA for 2 days before supernatants were collected for ELISA and cells for proliferation assay (see Fig. 6). Cultures without CAS-STA (Control) were also run in parallel.
tios of all challenged mice at all detected time points were higher than in naive mice (Fig. 7). Supernatants of cultured splenic cells also manifested the same bias toward a Th1-type response, especially when CAS-STA was supplemented to the culture (Fig. 7).

Infiltration of Lymphocytes and Immunoglobulin Deposition in Infected Corneas

When measured with immunohistochemistry, normal corneas did not show any deposition of IgA or IgG, nor did they contain any CD4\(^+\) or CD8\(^+\) lymphocytes (Fig. 8). Corneas with primary CaK did not contain CD4\(^+\) or CD8\(^+\) cells in the diseased regions, nor did they demonstrate antibody deposition at day 7 after intrastromal fungal injection. However, in the corneas that developed secondary CaK after pre-FK, pre-PI, or pre-AI, high levels of IgG and IgA and of CD4\(^+\) and CD8\(^+\) lymphocytes were found in the stroma of affected areas (Fig. 8).

**DISCUSSION**

The intrastromal injection model was chosen in this study to avoid the use of immunosuppressive agents usually needed for standard topical infections, to maximize the accuracy and consistency of loads of applied spores, and to ensure that the nonspecific barrier function of corneal epithelium does not
interfere with the outcome or interpretation of the results. By optimizing the dose of fungi used, we were able to induce CaK in all corneas without causing perforation, and all the corneas regained transparency within 3 weeks (Figs. 2, 3), allowing us to study the long-term effect of primary CaK on the immune compartment of the animals. To our knowledge, this is the first report of the adaptive immune response induced by FK.

First, we found that previous CaK disease, pulmonary infection, and active immunization all resulted in resistance to subsequent intrastromal fungal challenge, reflected by milder keratitis and faster recovery than in primary CaK cases or in age-controlled mice (Figs. 3, 4). At the same time, these primed mice produced more pathogen-specific immunoglobulins (especially IgG; Fig. 5) and more cytokines (especially IFN-γ; Fig. 7), implying that a memory immune response had been mounted. At the same time, marked CD4⁺ and CD8⁺ lymphocyte infiltration and extensive IgA/IgG deposition were observed in the stromal layers of corneas after secondary CaK in the primed mice, especially in corneas that had developed CaK twice (Fig. 8). Therefore, we had good reason to believe that the memory-type response to CaK in the primed mice was mediated by adaptive immune system components. Moderate

**Figure 8.** Immunostaining of corneas for IgA and IgG (upper) or CD4 and CD8 (lower). Corneas were removed from mice at day 7 after CaK induction and were frozen in OCT. Continuous cryosections were prepared, and neighboring sections were used for staining with IgA plus IgG or CD4 plus CD8. Sections were observed under G-2A mode for TRITC or PE labeling and B-2A for FITC labeling. Exposure time was set at 1 second, when pictures were taken to minimize the autofluorescence of corneas. Ongoing ulceration in the stroma showed universal fluorescence, whereas lymphocytes manifested dotted staining.
elevation of IgA levels in serum and intense IgA deposition in corneas of mice that recovered from secondary CaK (Fig. 8) confirmed the involvement of IgA in such an immune response. Finally, staining of cellular structures with IgA, but not IgG (Fig. 8), implied that a portion of the corneal IgA might be produced locally by infiltrating B lymphocytes, whereas IgG is recruited to the infectious region from other locations. It was reported that after topical challenge with *Pseudomonas aeruginosa*, IgA was secreted locally into tears without systemic production of IgA.\(^{17}\) Our data, however, that the production of IgA in splenic cells culture was also increased (Fig. 5) implied that IgA could be produced in central lymphoid organs (e.g., spleen in this case) and during corneal infection with *C. albicans*.

By comparing the changes in IFN-γ and IL-4 levels in serum and splenic culture medium, we noticed that the antifungal response in this CaK model was Th1-type biased (Fig. 7). This is consistent with other observations that the Th1-type response mediates the resistance to systemic *C. albicans* infection.\(^{18,19}\) Our data also showed that male and female mice responded to fungal challenge in exactly the same manner, as indexed by clinical scoring (Fig. 4), antibody production (Fig. 5), splenocyte proliferation (Fig. 6), and cytokine production (Fig. 7).

In the field of ocular infection, the adaptive immune response has long been overlooked.\(^{20}\) This study shows that various priming protocols induce immunologic memory and endorse the animals with higher resistance to the same strain of fungus. Thus, we propose that active immunization with killed or attenuated fungal preparations might help to prevent life-threatening infections and sight-threatening FK in susceptible persons such as patients with HIV/AIDS, who are at very high risk for fungal keratitis.\(^{21–23}\) A drawback of this study was that it did not establish the molecular mechanisms underlying the initiation or maintenance of adaptive immunity in the CaK. A recent work by Yuan et al.\(^ {24}\) sheds light on this area by profiling the responding genes in corneas with *C. albicans* keratitis. Consistent with our findings, Yuan et al.\(^ {24}\) showed that T-cell receptor, B-cell receptor, and TLR pathways are among the signaling pathways significantly upregulated during *C. albicans* keratitis.\(^ {24}\) Investigating these pathways and other immune-related pathways, such as chemokine or leukocyte extravasation, might be a good start toward identifying the molecular basis of specific immunity in the pathogenesis of FK. In fact, it has been shown that many corneal cells express various TLRs,\(^ {25,26}\) and MyD88 is activated through TLR4 in *Candida* keratitis.\(^ {27}\)

In summary, we observed a Th1-type specific immune response and an immunologic memory in mice with *C. albicans* keratitis. Previous contact with fungal preparation, either live or killed, enhanced the resistance of these mice to subsequent corneal challenge with the same fungus. More exhaustive studies using mice lacking specific components (e.g., nude mice for T-cell deficiency, μMT mice for B-cell deficiency, or SCID mice for both T- and B-cell deficiency) are necessary to further clarify whether a cellular or humoral immune response dominates in this situation. This work proposes that the role of adaptive immunity should be also considered when the pathogenesis, treatment, or prevention of FK is studied.

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**References**


