TEMPORAL RECRUITMENT OF NEUTROPHILS AND EOSINOPHILS TO THE SKIN IN A MURINE MODEL FOR ONCHOCERCAL DERMATITIS

ERIC PEARLMAN, CHRISTINE A. GARHART, DAVID J. GRAND, EUGENIA DIACONU, ELLEN R. STRINE, AND LAURIE R. HALL

Division of Geographic Medicine, Case Western Reserve University, Cleveland, Ohio

Abstract. The parasitic helminth *Onchocerca volvulus* causes ocular onchocerciasis (river blindness) and onchocercal skin disease. To understand the immunologic basis for early stage skin disease, we developed a model in which C57Bl/6 mice were immunized subcutaneously and injected intradermally (in the ear) with soluble *O. volvulus* antigens (OvAg). We found that ear thickness increased significantly after intradermal injection of OvAg and remained elevated for at least 7 days. Dermatitis was dependent on prior immunization, and was associated with an intense cellular infiltrate in the dermis. Neutrophils were the predominant inflammatory cells in the dermis 12 hr after intradermal injection, with only occasional eosinophils present. Conversely, increased ear thickness at later time points was associated with eosinophils, and neutrophils were only rarely detected. Both cell types were present at intermediate time points. These data indicate that recruitment of neutrophils and eosinophils to the skin is temporally regulated.

An estimated 17.7 million individuals are infected with the parasitic nematode *Onchocerca volvulus*, the causative organism of river blindness. In addition to ocular disease, the parasite larvae (microfilariae) in the skin can induce severe dermatitis, and an estimated 8.6 million infected individuals live in regions of Africa outside of the Onchocerciasis Control Program area where parasite strains are associated with skin disease. The impact of onchocercal skin disease on both the health of infected individuals and on the economy of the local communities is significant. In addition, onchocercal skin disease is a focus of the African Program for Onchocerciasis Control.4,5

In the current study, we used a murine model that reproduces clinical and histologic features of early stage onchocercal skin disease to examine underlying pathogenic mechanisms. We demonstrate that the presence of neutrophils and eosinophils in onchocercal skin disease is temporally regulated, with neutrophils preceding and being replaced by eosinophils.

MATERIALS AND METHODS

**Animals.** C57Bl/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME), and housed under microisolator conditions in the Animal Resource Center at Case Western Reserve University.

**Antigens.** *Parasite antigens.* Subcutaneous nodules containing adult *O. volvulus* parasites were surgically removed from patients at the Kumba Medical Research Station in Cameroon, and were provided by Dr. Peter Enyong (Kumba, Cameroon) and Dr. Sara Lustigman (New York Blood Center, New York, NY). Adult worms were isolated after collagenase digestion of the nodular material as described. Parasites were homogenized using a tissue grinder, and the extract was centrifuged to remove insoluble material. The supernatant containing soluble *O. volvulus* antigens (OvAg) was passed through a 0.2-μm syringe filter, and protein concentration was adjusted to 1 mg/ml.

**Mycobacterial antigens.** A soluble protein extract of *Mycobacterium tuberculosis* (purified protein derivative [PPD]) was a kind gift from Dr. Richard Silver (Case Western Reserve University).

**Immunization and intradermal injections.** Mice were sensitized to parasite antigens by 3 weekly subcutaneous immunizations with 10 μg of OvAg or PPD in adjuvant containing 10% squalene, 0.4% Tween 80, and 1% pluronic acid in saline (Hanks’ balanced salt solution [HBSS], Gibco-BRL, Gaithersburg, MD) at a 1:1 ratio as described. For injections into the ear, a 27-gauge needle was used to make a tangential opening in the epidermis, and a 30-gauge syringe (Hamilton Co., Reno, NV) containing 10 μg of soluble *O. volvulus* antigens or PPD in 10 μl of saline was injected into the opening in the ear pinna. Since there is not a clear delineation between the dermis and the subcutaneous tissue at this site, it is likely that antigen also entered subcutaneous tissue. Contralateral tissues were injected with HBSS.

**Ear thickness** was determined using a low-force micrometer with a 0.01-mm scale (APIS Instruments, Cleveland, OH). Ears were measured three times and the mean score was determined. The site of injection was marked with indelible ink throughout the experiment to ensure reproducibility of ear thickness measurements, and for correct orientation for histologic analysis.

**Histology and immunohistochemistry.** The details of the staining procedure for neutrophils and eosinophils have been described elsewhere. Briefly, ears were removed and fixed in 10% formaldehyde for at least 24 hr, followed by processing in a Tissue-Tek VIP tissue processor (Sakura, Los Angeles, CA). To identify eosinophils, ear sections were immunostained using rabbit antiserum to murine eosinophil major basic protein (kindly provided by Drs. Kirsten Larson and Gerald Gleich, Mayo Clinic, Rochester, MN). For neutrophils, ear sections were stained with rat monoclonal antibody 7/4 (Serotec, Oxford, United Kingdom), which reacts specifically with murine neutrophils.

**Antibody to eosinophilic major basic protein and monoclonal antibody 7/4** were diluted 1:1,000 and 1:100, respectively, in 1% fetal calf serum in 0.05 M Tris-buffered saline, pH 7.6, and slides were incubated at room temperature in a humid chamber for 2 hr. Biotinylated goat anti-rabbit immunoglobulin (Dako, Carpinteria, CA) diluted 1:200 or prediluted Rat Link (BioGenex, San Ramon, CA) was added for 30 min followed by a similar incubation with prediluted alkaline phosphatase–conjugated streptavidin (BioGenex).
PATHOGENESIS OF ONCHOCERCAL SKIN DISEASE

FIGURE 1. Development of *Onchocerca volvulus* antigen–induced dermatitis. A, C57Bl/6 mice were given three weekly subcutaneous immunizations with *O. volvulus* antigens. One week after the last immunization, animals were injected intradermally (in the ear) with either *O. volvulus* antigens or with saline, and ear thickness was measured. Each data point is the mean ± SD of 5 mice per group. Data are representative of three repeat experiments. B, C57Bl/6 mice were either immunized as described above or left unimmunized. Ears were then injected with *O. volvulus* antigens or with saline, and ear thickness was determined 7 days later. Data are the mean ± SD of 4 mice per group.

Positive reactivity was detected using Vector Red Substrate (Sigma, St Louis, MO), followed by counterstaining with modified Harris’ hematoxylin (Richard-Allen, Kalamazoo, MI).Slides were masked prior to examination, and at least 3 high-power fields at the site of the injection were counted.

**Statistical analysis.** Statistical analysis was performed using Student’s *t*-test. A *P* value < 0.05 was considered significant.

RESULTS

**Development of OvAg-induced dermatitis.** To determine if intradermal injection of *O. volvulus* antigens induces an inflammatory response in C57Bl/6 mice, animals were immunized and injected intradermally with OvAg. Increased ear thickness was used as a measure of inflammation. As shown in Figure 1A, ear thickness was increased after 24 hr compared to day 0, was further elevated at 48 hr, and remained high for 7 days. Intradermal injection of saline did not induce an inflammatory response at any time. In addition, ears of unimmunized mice did not increase in thickness after intradermal injection of parasite antigens (Figure 1B). These data indicate that development of murine onchodermatitis requires sensitization and local deposition of parasite antigens.

**Histologic features of OvAg-induced dermatitis.** To determine the nature of the inflammatory response in the skin, ears from immunized animals that were injected with either saline or OvAg were recovered after 72 hr, and 5-μm tissue sections were examined after staining with hematoxylin and eosin. Histologic examination of ears injected with OvAg revealed an intense inflammatory infiltrate at the site of injection in comparison with saline-injected ears (Figure 2). This observation is consistent with the notion that OvAg-

FIGURE 2. Histologic features of *Onchocerca volvulus* antigen–induced dermatitis. Mice were immunized with soluble *O. volvulus* antigens (OvAg) and injected intradermally with saline or OvAg. Three days after intradermal injection, animals were killed, and tissue was sectioned and stained with hematoxylin and eosin. A, saline-injected skin. B, OvAg-injected skin. Sections are representative of 5 mice per group in three separate experiments. (Magnification × 100.)
induced onchodermatitis is due to inflammatory cell migration into the skin. Examination at higher magnification indicated that most of the inflammatory cells were polymorphonuclear granulocytes.

**Temporal recruitment of eosinophils and neutrophils in OvAg-mediated dermatitis.** To differentiate and estimate the numbers of granulocytes in the skin at different time points after injection of OvAg, serial tissue sections collected at different times were immunostained with a monoclonal antibody for neutrophils (7/4) and with sera for eosinophil major basic protein. As shown in Figures 3 and 4, neutrophils were detected as early as 12 hr after intradermal injection of OvAg, whereas only occasional eosinophils were present. In contrast, eosinophils were the predominant cell type in the skin 7 days after injection. Both cell types were present in dermis examined at intermediate time points. These data indicate a biphasic recruitment of inflammatory cells into the dermis in OvAg-mediated onchodermatitis.

**Specificity of OvAg-induced dermatitis.** To determine if temporal recruitment of neutrophils and eosinophils to the skin is specific for *O. volvulus* antigens, mice were immunized and injected intradermally with mycobacterial PPD. Previous studies demonstrated that immunization with PPD induces a distinct cellular immune response from parasite antigens, and that injection into the cornea induced only mild keratitis compared with OvAg. In the current study, we found that ear thickness increased from 0.43 mm to 0.51 ± 0.41 (mean ± SD) on day 3 and to 0.5 ± 0.45 mm on day 7 after injection with PPD compared with OvAg-im-
mumized and challenged mice (0.63 and 0.6 on days 3 and 7, respectively). However, in contrast to OvAg-treated animals, the cellular infiltrate was primarily mononuclear, with few eosinophils or neutrophils.

**DISCUSSION**

Adult *O. volvulus* females have an estimated reproductive life of 11–13 years, and release an average of 1,000 microfilariae per day into the skin.12 While alive, the parasitic larvae generally do not induce an inflammatory response; however, larval death occurring either by natural attrition or after chemotherapy stimulates a localized inflammatory response that can result in development of dermatitis. Several chronic manifestations of onchocercal skin disease have been described, including acute and chronic papular onchodermatitis, lichenified onchodermatitis (sowda), dermal atrophy, and depigmentation.13 In contrast, acute papular onchodermatitis is an early manifestation of onchodermatitis and is characterized by the formation of papules that are intensely pruritic.13,14 Acute papular onchodermatitis may be a result of an inflammatory response to parasites dying by natural attrition, since histologic examination shows dead and dying larvae and infiltration of neutrophils and eosinophils.14

Ackerman and others demonstrated the presence of eosinophils and release of eosinophil major basic protein in the skin after systemic administration of the anthelmintic diethylcarbamazine (DEC).15,16 In addition, Stingl and others showed accumulation of eosinophils and deposition of major basic protein around parasites after topical application of DEC.17 However, while Gutierrez-Pena and others also showed deposition of eosinophil granule proteins 24 hr and 46 hr after application of topical DEC, including eosinophil cationic protein and eosinophil peroxidase, they found abundant deposition of neutrophil cytotoxic products including myeloperoxidase, lysozyme, elastase, lactoferrin, and defensin.18 These workers also noted that neutrophils were the prominent cell type in many of the lesions, especially the larger microabscesses.18 In the current study, we also found that neutrophils were prominent in the skin; however, we observed that these cells were present at early time points after exposure to parasite antigens, and were replaced by eosinophils after 7 days. The relative contribution of these cells to OvAg induced dermatitis will be determined by selective depletion of these cell types.

This biphasic recruitment implies that migration of these cells into the dermis is tightly regulated. Possible mechanisms include elevated expression of vascular endothelial cell adhesion molecules and chemotactic cytokines (chemokines). Expression of chemokines is elevated in cornneas of mice with OvAg-induced inflammatory responses (as a model for river blindness),19 and eosinophil recruitment to the cornea is significantly reduced in animals deficient in expression of the eosinophil chemokine eotaxin.20 The use of antibodies to neutralize these chemokines and block cell adhesion molecules along with the use of gene-targeted mice will allow assessment of their role in inflammatory cell recruitment in onchocercal dermatitis.

The observation that development of OvAg-mediated dermatitis required both systemic immunization and local injection of parasite antigens indicates that an active immune response is essential. These findings are consistent with our previous studies demonstrating that prior immunization is required in a murine model for ocular onchocerciasis in which parasite antigens are injected into the cornea.21 In that study, we found that immunization does not develop in the absence of T cells, and that immunization with OvAg induces a selective T helper type 2 response with elevated interleukin-4 (IL-4) and IL-5 production by splenocytes and elevated serum IgE.7,21 The observation that neutrophil migration to the skin precedes that of eosinophils was also noted in OvAg-mediated corneal inflammation.8

Although occasional inflammatory cells are detected in the epidermis in OvAg-injected animals, the inflammatory response was primarily in the dermis, which is the site of deposition of parasite antigens. This differs from patients with DEC-induced lesions, in which onchocercal skin lesions are detected in both the dermis and epidermis.15–18 Epidermal lesions are probably due to migration of parasites into the epidermis prior to larval death and deposition of larval antigens. Other studies have used live microfilariae rather than soluble antigens in models of airway hyperresponsiveness induced by *Brugia*22–24 and vaccine-induced immunity to *O. lienalis*.25–27 As with the current study, these reports showed responses consistent with selective Th2 induction, notably tissue eosinophilia. Injection of *B. malayi* microfilariae into the skin or cornea of sensitized animals also showed temporal recruitment of neutrophils and eosinophils as described for OvAg (Pearlman E and others, unpublished data). Together with the finding that immunization and injection of PPD induces inflammatory cell migration to the skin, these observations support the notion that it is not the capacity of this parasite to induce a Th2 response or stimulate inflammatory cell recruitment to the skin that is unique to this organism; rather it is the innate predisposition of *O. volvulus* to invade the skin of infected individuals that ultimately leads to development of dermatitis in this disease.

In conclusion, the murine model for onchodermatitis described herein will allow further examination of the immune responses that underlie this important disease, especially with respect to early manifestations of onchocercal skin disease.

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Authors’ address: Eric Pearlman, Christine A. Garhart, David J. Grand, Eugenia Diaconu, Ellen R. Strine, and Laurie R. Hall, Division of Geographic Medicine, Case Western Reserve University School of Medicine, 2109 Adelbert Road, Cleveland OH 44106.

Reprint requests: Eric Pearlman, Division of Geographic Medicine, Case Western Reserve University School of Medicine, 2109 Adelbert Road, Cleveland OH 44106.

**REFERENCES**


