method to screen for glaucoma therapy compounds that could be directed at the outflow pathway.

References


Mice Deficient in Tumor Necrosis Factor Receptors p55 and p75, Interleukin-4, or Inducible Nitric Oxide Synthase Are Susceptible to Endotoxic-Induced Uveitis

Justine R. Smith,¹ Prue H. Hart,² Douglas J. Coster,³ and Keryn A. Williams¹

PURPOSE. To investigate the roles of tumor necrosis factor-α (TNF-α), interleukin-4 (IL-4), and inducible nitric oxide synthase (iNOS) in endotoxin-induced uveitis (EIU) using gene knock-out mice.

METHODS. Mice (C57BL/6 x 129) either of normal phenotype or deficient in the genes encoding one or both tumor necrosis factor receptors (TNFR p55 and TNFR p75), IL-4, or iNOS were given footpad injections of 400 µg Escherichia coli lipopolysaccharide. Animals were killed 24 hours later, and infiltrating cells were counted on 5-µm ocular cross-sections through the optic nerve.

RESULTS. All abnormal mouse phenotypes were susceptible to EIU. Yet, TNFR p55 and IL-4 gene knock-out mice experienced less ocular inflammation than control animals (P = 0.021 and 0.007, respectively), whereas disease was not reduced for iNOS-deficient mice. Mice deficient in TNFR p55 and TNFR p75 experienced milder EIU than mice lacking TNFR p75 alone (P = 0.046).

CONCLUSIONS. Mice deficient in TNFR p55 and TNFR p75, IL-4, or iNOS retain the susceptibility to EIU, but TNF-α and IL-4 influence the influx of inflammatory cells to the eye during this disease. (Invest Ophthalмol Vis Sci. 1998; 39:658-661)

Acute anterior uveitis causes distressing ocular symptoms and, when recurrent, may lead to sight-threatening disease, including cataract, glaucoma, and cystoid macular edema. Current therapy involves relatively nonspecific suppression of inflammation using topical corticosteroids. Although frequently effective, these agents may induce cataract or raise intraocular pressure, and patients are prone to microbial infection and rebound inflammation after drug withdrawal. The basic mechanisms operating in acute anterior uveitis have not yet been clarified. Little human material is available for research, but animal models of the disease provide opportunities to study the pathogenic process and devise more specific treatment options. In one well-established model known as endotoxin-induced uveitis (EIU), rodents injected with bacterial lipopolysaccharide develop an evanescent form of acute anterior uveitis. Cytokines are presumed to drive uveal inflammation. Studies in the rat have implicated tumor necrosis factor-α (TNF-α) as a critical early mediator of uveitis. Reverse transcription-polymerase chain reaction (RT-PCR) detects an increase in TNF-α gene expression within 1 hour of systemic endotoxin injection, and the TNF-α mRNA has been localized to histiocyte-like cells by in situ hybridization. The expressed product has been detected in the anterior segment before and during EIU by bioassay. Macrophages and monocytes, the major producers of this cytokine, respond to stimuli such as lipopolysaccharide, interleukin-1 (IL-1), and TNFα itself. The multiple inflammatory activities of TNF-α are signaled through two distinct cell surface tumor necrosis factor receptors, designated TNFR p55 and TNFR p75, respectively.

From the Departments of ¹Ophthalmology and ²Microbiology and Infectious Diseases, Flinders University of South Australia, Bedford Park, Australia. Supported in part by The National Health and Medical Research Council, The Ophthalmic Research Institute of Australia, and The Flinders Medical Centre Foundation. Submitted for publication June 12, 1997; revised October 31, 1997; accepted November 10, 1997. Proprietary interest category: N. Reprint requests: Justine R. Smith, Department of Ophthalmology, Flinders Medical Centre, Bedford Park, South Australia 5042, Australia.
Interleukin-4 (IL-4) is an immunomodulatory cytokine synthesized by several cell types, including T lymphocytes. Stimulatory and inhibitory effects on lymphocytes are recorded for IL-4, but macrophage and monocyte activity is generally inhibited. Although macrophages and neutrophils are generally considered to be the important players in EIU, recent evidence indicates that T lymphocytes also participate in the pathogenesis of this disease. The role of IL-4 in EIU has not previously been explored, but secretion of this T cell product may logically be expected during EIU. Furthermore, it may be anticipated that IL-4 would downregulate the inflammation.

Accumulating evidence suggests that nitric oxide (NO) is a key secondary mediator in EIU. Nitric oxide is an oxygen-free radical released from l-arginine by either constitutive or inducible nitric oxide synthase (cNOS or iNOS). Synthesis of iNOS can be induced by lipopolysaccharide or such cytokines as TNF-α and IL-1. High levels of nitrite are detected in the ocular fluids of Lewis rats with EIU, and synthesis of iNOS mRNA by the anterior uveal epithelia and retina, as well as infiltrating cells, is supported by RT-PCR and in situ hybridization experiments. Drugs that inhibit NOS activity significantly reduce the severity of EIU.5 6

Gene targeting, the homologous recombination of chromosomal DNA sequences with introduced DNA sequences, has recently been used to generate knockout mice deficient in one or more gene products. We wanted to determine whether TNF-α or iNOS was obligatory to the pathogenesis of murine EIU, using C57BL/6 x 129 mice deficient in TNFR p55, TNFR p75, or iNOS. If it were possible to identify a molecule critical to the disease process, specific treatment could sensibly be targeted against that mediator. Additionally, we wanted to examine the course of EIU in C57BL/6 x 129 IL-4 knockout mice. An alternative therapeutic strategy could involve the inhibition of inflammation by an immunomodulatory cytokine such as IL-4.

METHODS

Animals
Male and female mice (8 weeks or younger) were used. Animals were housed at 21°C and 50% humidity in a 12-hour light–12-hour dark cycle and fed water and dried ration (New Joint Stock; Ridley Agriproducts, Murray Bridge, Australia). Experimental protocols were developed in accordance with the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All procedures and euthanasia were carried out under halothane (Fluothane; Zeneca, Macclesfield, United Kingdom) inhalation anesthesia.

TNFR p55 knockout mice, IL-4 knockout mice, and the normal phenotype C57BL/6 x 129Sv controls were obtained from the offices of H. Blüthmann (Hoffmann-La Roche, Basel, Switzerland). J. Peschon (Immunex, Seattle, WA) generated TNFR p75-deficient and TNFR p55/p75-deficient mice, each on a C57BL/6 x 129SvEv background. The normal phenotype C57BL/6 x 129SvEv mouse from which these mutants were generated was unavailable to us. J. MacMicking, C. Nathan, and J. Mudgett of Cornell University Medical School (New York, NY) and Merck Research Laboratories (Rahway, NJ) supplied iNOS knockout mice and C57BL/6 x 129SvEv controls. Southern blot analysis of tail DNA was used to confirm gene deficiency in the local colonies. All C57BL/6 x 129 mice were from F2 or subsequent breedings.

Induction of Endotoxin-Induced Uveitis
Lipopolysaccharide from E. coli O55:B5 was obtained from Sigma Chemical (St. Louis, MO). Preliminary studies indicated that C57BL/6 x 129 mice were susceptible to EIU, with 400 μg lipopolysaccharide producing maximal inflammation. In a total of seven experiments, gene knockout mice and control animals of normal phenotype were given 400 μg lipopolysaccharide, dissolved in a sterile, nonpyrogenic 0.9% saline solution, as a 40-μl subcutaneous injection in the right hind footpad.

Assessment of Uveal Inflammation
Animals were euthanatized 24 hours after the lipopolysaccharide injection, and the eyes were immediately enucleated. One eye from each mouse was fixed in 10% buffered formalin for a minimum of 24 hours and subsequently embedded in paraffin wax. Tissue cross-sections, including the optic nerve head, were cut 5-μm thick and stained with hematoxylin and eosin. The total number of inflammatory cells infiltrating the anterior and posterior segments of the eye was determined at 400× magnification. Cell counts for gene knockout mice and their respective controls were compared using the Mann-Whitney test, corrected for ties. Five gene knockout mice of each abnormal phenotype were not administered endotoxin but were killed in an identical manner. Eyes of these animals were handled as described above and served as the controls for the euthanasia and processing procedures.

RESULTS
In the normal C57BL/6 x 129 mouse, EIU was manifest as both anterior and posterior segment inflammation (Fig. 1). The majority of infiltrating cells were polymorphonuclear leukocytes, but mononuclear cells were also present. Red blood cells were absent. Inflammatory cells infiltrated the vitreous, particularly in the region of the optic nerve head, and, in some cases, cells were observed within the substance of the intracocular portion of the optic nerve. Smaller numbers of cells were also seen in the anterior and posterior chambers, accompanied by eosinophilic material presumed to represent fibrin clot. Cornea, sclera, lens, retina, and choroid were not involved.

All gene knock-out phenotypes retained susceptibility to EIU. Histologic features of the disease were identical with those observed in the normal mice, yet TNFR p55 knockout mice developed relatively less severe EIU than the control animals (Fig. 2A), and EIU was milder in TNFR p55/p75-deficient mice than in TNFR p75-deficient mice (Fig. 2B). IL-4 knockout mice experienced milder ocular inflammation than normal controls (Fig. 2A). iNOS-producing and iNOS-deficient mice were equally susceptible to EIU (Fig. 2C). No ocular inflammation was detected among the gene knock-out mice euthanatized but not injected with lipopolysaccharide.

The total number of infiltrating cells was found to vary widely among mice of any given phenotype. Furthermore, for the C57BL/6 x 129 control mice, there was a statistically significant difference in cell counts for animals of the C57BL/6 x 129Sv (Fig. 2A; normal) and C57BL/6 x 129SvEv (Fig. 2C; normal) substrains (P < 0.0001). There was no statistically
for all phenotypes, including normal, and is characterized by IL-4, or iNOS, these mice do not become completely resistant to the disease. The histologic picture of EIU is identical for all phenotypes, including normal, and is characterized by an accumulation of neutrophils in the aqueous humor and the posterior vitreous, as previously described for other mouse strains.1,7 Uveal inflammation does not occur spontaneously in mice with the respective gene deletions. Our data show that none of the molecules studied, TNF-α, IL-4, or iNOS, is absolutely necessary for the development of endotoxin-induced uveitis in the mouse.

Mice deficient in TNFR p55 experienced less inflammation than normal controls, and TNFR p55/p75 knock-out mice showed less inflammation than TNFR p75-deficient mice. These results provide further evidence that TNF-α influences the influx of inflammatory cells to the eye during murine EIU. In addition, our data imply that TNFR p55 participates in this activity. TNFR p55 is the more biologically active of the two receptors, but TNFR p75 has a 5-fold higher affinity for TNF-α. When levels of TNF-α are low, TNFR p75 facilitates TNFR p55 activity, possibly by a ligand-passing mechanism or as a result of intracellular kinase activation. However, TNF-α is not the sole influence on cellular influx during EIU because inflammation occurs in mice lacking TNFR p55 and TNFR p75.

Interestingly, IL-4 knockout mice developed significantly less severe EIU than C57BL/6 x 129 control animals in our experiments. Ramanathan and colleagues8 recently described a similar pro-inflammatory effect of this cytokine in experimental autoimmune uveoretinitis (EAU) in the Lewis rat. Recombinant IL-4 aggravated experimental autoimmune uveoretinitis, and the effect was reversed by the administration of anti-IL-4 antibody. Cytokine assays suggested that this activity of IL-4 was mediated by TNF-α and possibly interferon-γ and nitric oxide. Additional inflammatory actions of IL-4 in EIU may include the expression of the adhesion molecules vascular cell adhesion molecule-1 and sialoadhesin; the upregulation of major histocompatibility class II molecules on macrophages and monocytes; and the production of interferon-γ by lymphocytes and the production of IL-6 by macrophages.

Although production of iNOS is evident during EIU,4 mice lacking the gene encoding this enzyme developed EIU identical to that observed in iNOS producers. Our result implies that if nitric oxide is an essential mediator of EIU, it can be produced in sufficient quantities by cNOS. Studies using selective inhibitors have suggested that cNOS plays a critical role in the earliest stages of EIU, whereas iNOS participates later in the course of disease.5,6 Alternatively, in the face of reduced nitric oxide synthesis in EIU, other mediators, such as cytokines or eicosanoids, may replace the inflammatory activities of this molecule.

Within any group of gene-deleted or normal phenotype C57BL/6 x 129 mice, infiltrating cell numbers varied widely. Knock-out mice are generated as germ-line chimeras: 129 embryo-derived stem cells are introduced into C57BL/6 x 129 mouse blastocysts. As a consequence, unlinked loci from C57BL/6 and 129 strains are randomly segregated in the progeny of these animals. The ocular inflammation induced in a given mouse by systemic lipopolysaccharide injection is strain dependent.1 Consequently, an unknown proportion of variation in the inflammatory cell counts relates to variable contributions of the C57BL/6 and 129 backgrounds rather than to the presence or absence of a particular gene product. The high degree of genetic variation within 129 substrains, and hence embryo-derived stem cell lines, may further complicate the results of experiments that rely on a standard inbred background.9 This is illustrated by the statistically significant difference in inflammatory cell counts for normal C57BL/6 x 129Sv and C57BL/6 x 129SvEv mice. To minimize the variation related to the genetic background, control animals were obtained directly from the original sources. However, we recognize the limitations of using such crossed background gene knock-out mice for quantitative comparisons. Crossing all gene deletions to a single, well-characterized background strain would solve this problem.

All gene knock-out phenotypes investigated were susceptible to EIU. Furthermore, for any given abnormal phenotype, some animals experienced more inflammation than a number of the controls. Mice lacking the genes for other inflammatory cytokines or receptors, including IL-1 receptor10 and IL-6,11 are also reported to develop EIU. In other words, no single molecule has been found to be obligatory to the pathogenesis of EIU.
in the mouse. Redundancy in inflammatory mediators may explain this observation. Various secondary mediators have been identified during EIU, and there is a simultaneous appearance of mRNA coding for multiple cytokines before the onset of disease. It follows that treatment targeting one inflammatory molecule is unlikely to be successful. To induce disease remission, it may be necessary to inactivate several molecular mediators.

Acknowledgments

The authors thank Jennifer Clarke, Michelle Lewis, Shaun Roman, and Scott Standfield for expert advice; Raymond Yates for animal husbandry; Paul Stoll for computer graphics; and Jacques Peschon for a critical review of the manuscript.

References