Central Nervous System-Targeted Expression of the Complement Inhibitor scCrry Prevents Experimental Allergic Encephalomyelitis

Nathalie Davoust, Serge Nataf, Rachael Reiman, Michael V. Holers, Iain L. Campbell and Scott R. Barnum

*J Immunol* 1999; 163:6551-6556;
http://www.jimmunol.org/content/163/12/6551

**References**

This article cites 22 articles, 9 of which you can access for free at:
http://www.jimmunol.org/content/163/12/6551.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Central Nervous System-Targeted Expression of the Complement Inhibitor sCrry Prevents Experimental Allergic Encephalomyelitis

Nathalie Davoust,* Serge Nataf,* Rachael Reiman,* Michael V. Holers,† Iain L. Campbell,‡ and Scott R. Barnum²,*

Although generally thought of as a T cell-driven autoimmune disease, recent studies in experimental allergic encephalomyelitis (EAE), the animal model of multiple sclerosis, suggest a significant role for innate immune mechanisms. To address the possibility that the complement system plays a central role in these diseases, we developed a transgenic mouse with astrocyte-targeted production of a soluble inhibitor of complement activation, complement receptor-related protein y (sCrry). Here, we show that sCrry transgenic mice are either fully protected against EAE or develop significantly delayed clinical signs. These results indicate that complement activation may have an essential role in the pathogenesis of the disease and that complement-mediated events may occur early during the effector phase of EAE. Furthermore, this work underscores the importance of humoral immunity in amplifying a T cell-initiated pathogenic process. The Journal of Immunology, 1999, 163: 6551–6556.

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the CNS in which the pathophysiology remains unclear. Primary lesions are characterized by areas of demyelination associated with infiltration by mononuclear cells and deposition of complement components including the membrane attack complex (1, 2). In both MS and its animal model experimental allergic encephalomyelitis (EAE), it is thought that infiltrating CD4⁺ T cells initiate an inflammatory process and elicit other immune effectors to mediate tissue destruction (1, 3, 4). However, recent studies indicate that in MS lesions, demyelination may occur in the absence of infiltrating T cells (1). It was also recently shown that B cell-deficient mice are susceptible to EAE and present all the clinical and histological features observed in wild-type mice (5). Furthermore, studies utilizing cytokine transgenic or knockout mice have demonstrated that proinflammatory cytokines, such as IFN-γ and TNF-α, may not be involved in disease development or progression and may actually be protective in EAE, although this remains controversial (6, 7). These data suggest that other immune effectors must be involved in initiation and perpetuation of this demyelinating disease. The complement system, an important component of innate and adaptive immunity, has been implicated in contributing to the inflammation and cellular destruction characteristic of MS and EAE. However, the actual pathogenic role of complement in demyelinating diseases remains poorly understood (2, 8, 9). Transient inhibition of complement activation in EAE results in protection from disease and therefore supports a pathogenic role for complement in disease development (10–12). However, these studies were performed using an Ab-mediated form of EAE, a highly acute disease, which is not representative of the disease course in MS. Further attempts to clarify the role of complement in a chronic disease setting have been limited by the lack of efficient and species-specific complement inhibitors. To overcome these limitations and to better understand the role of complement in EAE, we generated transgenic mice with astrocyte-targeted expression of a soluble, mouse-specific complement inhibitor, complement receptor-related protein y (Crry). Crry belongs to the large family of regulators of complement activation molecules and its soluble form (sCrry) was recently shown to block the activation of both the classical and alternative complement pathways in vitro and in vivo (13, 14). In the present study, we used a model of active EAE, induced with myelin oligodendrocyte glycoprotein (MOG) peptides, and found that sCrry transgenic mice are either fully protected from EAE or present a significantly delayed onset of the disease when compared with their wild-type littermates.

Materials and Methods

Generation of GFAP-sCrry transgenic mice

The cDNA encoding sCrry (14) was subcloned into the NosI site of the pGF.GH construct containing the mouse glial fibrillary acidic protein (GFAP) promoter and the polyadenylation signal sequence from the human growth hormone (hGH) (15). The GFAP-sCrry fragment was excised with SfiI and EcoRI and injected into C57BL/6 × SJL/J fertilized eggs by staff of the transgenic core facility at the University of Alabama at Birmingham. Founders were identified by PCR amplification performed on tail-derived DNA using a Crry (5’-GAA-CTC-AAC-AAA-TGT-ACT-3’) and hGH (5’-TGG-GCA-CTG-GAG-TGG-CAA-3’) primer set which detects only sCrry-positive animals. The amplification products were confirmed as sCrry specific by Southern blot analysis (data not shown).

*Department of Microbiology, University of Alabama, Birmingham, AL 35294;
†University of Colorado Health Sciences Center, Denver, CO, 80262; and ‡Department of Neuropharmacology, The Scripps Research Institute, La Jolla, CA 92037

Received for publication August 24, 1999. Accepted for publication September 24, 1999.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by National Multiple Sclerosis Society Grant PP0551 (to S.R.B.) and National Institutes of Health Grants NS29719 (to S.R.B.), MH50426 (to L.L.C.), and AI31105 (to V.M.H.). S.N. is the recipient of an Advanced Postdoctoral Fellowship (FA 1306A) from the National Multiple Sclerosis Society.

2 Address correspondence and reprint requests to Dr. Scott R. Barnum, Department of Microbiology, Division of Clinical Immunology, University of Alabama, Birmingham, 701 19th Street South, LHR/141, Birmingham, AL 35294. E-mail address: sbarnum@uab.edu

3 Abbreviations used in this paper: MS, multiple sclerosis; Crry, complement receptor-related protein y; sCrry, soluble form of Crry; EAE, experimental allergic encephalomyelitis; GFAP, glial fibrillary acidic protein; hGH, human growth hormone; MOG, myelin oligodendrocyte glycoprotein; CSF, cerebrospinal fluid.
FIGURE 1. Generation of GFAP-sCry transgenic mice. A. A truncated Cry cDNA containing only the short consensus repeat regions was cloned in the NotI site of the pGF.GH vector and placed under control of the GFAP promoter. The polyadenylation sequence for the sCry gene was provided from the hGH. A schematic of the construct is shown. N, NotI; P, PvuI; S, SfiI; SCR, short consensus repeat; TM, transmembrane region; Cyto, cytoplasmic region. B. Expression of sCry mRNA was determined by RT-PCR using Cry and hGH primers. sCry mRNA was detected in RNA isolated from whole brain from the 5-7 and 5-9 founder mice used in this study, but not in RNA from wild-type mice or in the absence of RNA (H2O). C, Positive control (100 copies of the GFAP-sCry construct); NTG, nontransgenic mouse.

Western blot

Cerebrospinal fluid (CSF) and serum from control (n = 3) and transgenic mice (n = 3) were diluted in nonreducing SDS-PAGE loading buffer (125 mM Tris (pH 6.8), 4% SDS, and 20% glycerol) in the presence of protease inhibitors (0.3 mM aprotinin, 1 mM leupeptin, 1 mM pepstatin, and 1 mM EDTA). Samples were electrophoresed in a 7.5% SDS-polyacrylamide gel, and proteins were transferred to a nitrocellulose membrane. For the detection of Cry protein, blots were blocked for 1 h at room temperature in PBS (pH 7.5) containing 10% (w/v) nonfat dry milk, washed, and polyclonal rabbit anti-mouse Cry Abs (0.5 μg/ml) (14) were added for 1 h at room temperature. Blots were washed and then treated with 1/10,000 dilution of peroxidase-conjugated anti-rabbit IgG secondary Ab. The secondary Ab was detected using the Amersham chemiluminescence-based enhanced chemiluminescence detection kit (Amersham, Piscataway, NJ).

ELISA for determination of Cry levels

ELISA analysis was performed on supernatants of astrocyte cultures derived from control and sCry transgenic mice. The primary astrocyte cultures were obtained from the cortex of newborn mice as described previously (16) and maintained until confluency (8–10 days) in DMEM/F-12 supplemented with 10% heat-inactivated normal calf serum. For the ELISA, Dynatech Immulon II (Dynatech Laboratories, Chantilly, VA) 96-well plates were coated at 4°C overnight with 1 μg/well IgG purified from rabbit anti-mouse Cry polyclonal Ab (14). Plates were then washed four times with PBS and 0.05% Tween 20 and blocked for 1 h at room temperature with PBS and 1% BSA. The plates were washed four more times and then samples, diluted in PBS with 0.1% BSA, were added for a 1-h incubation. Plates were then decanted and washed four times before the addition of pretitrated anti-Cry mAb 10A2 coupled with biotin in PBS and 0.1% BSA. After a 1-h incubation, the plates were again washed four times. Streptavidin-HRP (Sigma, St. Louis, MO) diluted in PBS was then added for 30 min. After four washes, 2,2′-azino(3-ethylbenzthiazoline-sulfonic acid) (Boeringer Mannheim, Indianapolis, IN) activated in H2O2 was added. Plates were developed in the dark and the OD405 was measured with a Titertek Plus ELISA plate reader (ICN Pharmaceuticals, Costa Mesa, CA).

EAE induction and clinical evaluation

Mice were immunized using a mixture of MOG peptides 35–55 and 96–106. Both peptides were synthesized by standard 9-fluorenyl-methoxycarbonyl chemistry and were >95% pure as determined by reversed phase-HPLC (Research Genetics, Huntsville, AL). Mice were injected s.c. on day 0 and 7 with both peptides (150 μg each) emulsified in CFA. In addition, on days 0 and 2 postimmunization, mice were given pertussis toxin (500 ng) i.p.

Clinical signs of EAE were assessed daily using a standard scale (5) from 0 to 10, as follows: 0, no clinical signs; 1, loss of tail tone; 2, flaccid tail; 3, incomplete paralysis of one or two hind legs; 4, complete hind limb paralysis; 5, moribund; and 6, death.

Histological assessment

Mice were sacrificed by CO2 inhalation, brain and spinal cord were removed, and either immediately fixed with 4% formaldehyde and 2% glutaraldehyde or snap-frozen and kept at −80°C until examination. Spinal cords were either sectioned at 8 μm and stained with hematoxylin and eosin or embedded in Epon, sectioned at 1 μm, and stained with toluidine blue.

Immunohistochemistry

Tissue sections (8 μm) were prepared from EAE mice for each group of animals (control C57BL/6 × SJL, n = 3; sCry C57BL/6 × SJL, n = 3; control SJL/J, n = 3; and sCry SJL/J, n = 3). Sections were fixed in acetone for 6 min and then analyzed according to an immunoenzymatic staining method (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Briefly, tissues were blocked in PBS and 10% goat serum and then sequentially incubated with a rat monoclonal anti-mouse complement component C4 Ab (25 μg/ml) for 1 h (Accurate Chemicals and Scientific, Westbury, NY), a biotin-conjugated goat anti-rat IgG for 1 h (Jackson ImmunoResearch, West Grove, PA), and then a solution of 1% hydrogen peroxide for 15 min. After several rinses, the cells were treated with an avidin-biotinylated-peroxidase complex for 50 min at room temperature (Vectastain ABC kit; Vector), followed by 0.04% diaminobenzidine (Sigma) in PBS with 0.01% H2O2 for 10 min.

Results

To generate sCry transgenic mice, murine Cry cDNA, minus the transmembrane and cytoplasmic domains (14) was placed under the transcriptional control of a GFAP promoter (15) (Fig. 1A). This construct leads to astrocyte-specific production of sCry. Five founder lines were positive for the presence of sCry DNA. Of these founders, we used the 5-7 and 5-9 lines which expressed sCry at the mRNA level (Fig. 1B) and at the protein level as determined by Western blot analysis of CSF samples (Fig. 2A). The capacity of astrocytes to secrete sCry was further demonstrated by the detection of significantly elevated levels of sCry (p < 0.017) in the supernatants of astrocyte primary cultures derived from transgenic mice compared with control mice as assessed by ELISA (Fig. 2B). Heterozygous sCry mice appeared phenotypically normal, and their CNS did not show any morphological abnormality under macroscopic examination. As previously described for other transgenic mice using a GFAP promoter construct, we found a low transcript level for the sCry transgene in peripheral organs, such as gut, by RT-PCR (data not shown; Ref. 15). However, it should be noted that we did not detect sCry...
in serum of transgenic animals by Western blot or ELISA, suggesting that there is no significant sCrry protein synthesis outside the CNS (Fig. 2A).

To determine whether sCrry mice were protected from EAE compared with their wild-type littermates, we induced active EAE using MOG peptides (17). Because the sCrry transgenic mice were developed in a hybrid background (C57BL/6 × SJL/J), we chose to induce EAE using a mixture of two MOG peptides, MOG92–106, which is encephalitogenic for SJL/J (H-2D^d) mice, and MOG55–65, which is encephalitogenic for C57BL/6 (H-2D^b) mice. Using this protocol, we found that 95% of the wild-type C57BL/6 × SJL/J hybrid mice developed clinical signs of EAE accompanied by CNS inflammation and demyelination. As expected, the clinical course of the disease correlated with the MHC phenotype of the mice as determined by flow cytometry at the time of sacrifice. Thus, only the animals (Fig. 3A; Table I). Moreover, these mice did not have significant weight loss, whereas their wild-type MHC-matched littermates had a 10% weight loss starting at the clinical onset of disease (Fig. 3C). The 5-7 and 5-9 sCrry transgenic mice with a C57BL/6 MHC background (H-2D^d/H-2D^b or H-2D^d/H-2D^b) presented a statistically significant delayed onset of the disease when compared with their wild-type littermates (Fig. 3B; Table I). However, in these mice, EAE was otherwise similar to that observed in wild-type mice as assessed by maximum clinical scores and histopathological analysis.

Histopathological analysis of the spinal cords of 5-7 and 5-9 sCrry transgenic mice with the C57BL/6 or C57BL/6 × SJL/J MHC background showed significant cellular infiltration in the spinal cord similar to that observed in wild-type littermates (Fig. 4, A and B). Based on the level of disease observed at the end of the study, this observation is not surprising. In these mice, the spinal cord was infiltrated by mononuclear cells comprising CD4^+ T cells and monocytes (data not shown). Furthermore, these mice had levels of demyelination similar to those observed in wild-types control animals (data not shown). In contrast, sCrry transgenic mice with the SJL/J MHC background showed only very mild or no cellular infiltration compared with wild-type littermates (Fig. 4, C and D). The majority of any infiltrate seen in these mice was mononuclear, based on CD11b staining, and no CD4^+ T cells were observed in the spinal cord parenchyma (data not shown). These mice also had virtually no demyelination compared with the wild-type controls (Fig. 4, E and F).

To study complement deposition in the CNS of controls and transgenics during the course of EAE, immunohistochemistry was performed using an anti-C4 Ab. In analyzing brain sections obtained from each group of animals (Fig. 5), we found infiltrated lesions in the cerebellum of the control C57BL/6 × SJL/J and SJL/J MHC background mice (Fig. 5, A and D) as well as in the sCrry transgenic mice. In control mice, we observed a strong C4 staining at the edge of the lesions (Fig. 5, B and E), which was also seen, although to a lesser extent in the C57BL/6 × SJL/J sCrry transgenic mice (Fig. 5C). In sCrry transgenic mice with SJL/J MHC background, we did not detect any complement C4 deposition (Fig. 5F) on the few infiltrating lesions observed in the cerebellum.

Discussion
Our results clearly show that CNS-targeted expression of the complement inhibitor sCrry is efficient in blocking both inflammation and demyelination during MOG-induced EAE. The effects we observed are due to the intrathecal inhibition of complement activation since C4 deposition was largely undetected in the CNS of sCrry transgenic mice. The absence of antigenically detectable C4 deposition in sCrry mice is likely due to the enzymatic degradation of C4b by factor I, using sCrry as a cofactor (13, 14). Our data further demonstrate that complement-dependent events may play a pivotal role early in the effector phase of EAE. The fact that spinal cord infiltration was strikingly inhibited in sCrry transgenic mice, protected from EAE, suggests that trafficking of inflammatory cells into the CNS was blocked. Similarly, the delayed onset of the disease observed in sCrry transgenic mice developing EAE may indicate a delayed chemotaxis of leukocytes to the CNS. Indeed the anaphylatoxic fragments, C5a and C3a, both potent chemotactic factors, may chemoattract all the major cell types involved in EAE, including monocyte/macrophages, microglia, astrocytes as well as T cells, as recently demonstrated for C5a (19, 20). Aside from chemoattraction, C5a and C3a have also been shown to contribute to the induction of proinflammatory cytokines, chemokines as well as complement protein production, and adhesion molecule

---

**FIGURE 2.** Detection of sCrry protein in the CSF of GFAP-sCrry transgenic mice. A, Western blot analysis was performed on CSF and serum collected from wild type control (C) and sCrry-transgenic (TG) mice as described in Materials and Methods. A polyclonal anti-Crry Ab (0.5 μg/ml) was used for detection of sCrry. A Crry-IgG chimeric protein with a molecular weight of 160 was used as a positive control. Shown to the right of the blot are the positions of m.w. standards. B, Detection by ELISA of sCrry protein in the supernatant of primary astrocyte cultures derived from control (three independent cultures) and GFAP-sCrry transgenic mice (TG; three cultures derived from the 5-7 line and three cultures derived from the 5-9 line). The ELISA was performed as described in Materials and Methods. The bar shown in each group represents the mean sCrry level. The level of sCrry detected in astrocyte cultures from transgenic mice was significantly higher as assessed by Student’s t test (p < 0.017).
FIGURE 3. Clinical course of MOG-induced EAE in wild-type and 5-7 and 5-9 sCry transgenic mice. A, EAE was induced and scored in 5-7 and 5-9 sCry transgenic mice bearing a SJL/J MHC phenotype and in their wild-type MHC-matched littermates as described. B, Same as A except wild-type and 5-7 and 5-9 sCry transgenic mice with a C57BL/6 or C57BL/6 × SJL/J MHC background were analyzed. C, Weight changes in wild-type and sCry transgenic with an SJL/J MHC phenotype. Shown is the mean weight of all wild-type and all sCry transgenic mice during EAE.
expression (21–24). Because cytokine and chemokine-induced expression of adhesion molecules on endothelial and T cells is essential for T cell trafficking into the CNS (25), these additional functions of the complement anaphylatoxins may be critical in disease development. Thus, many of the early inflammatory mechanisms described in EAE and MS may be complement driven.

The failure of sCrry to completely protect C57BL/6 and C57BL/6 × SJL/J transgenic mice against EAE may be due to the more severe disease that develops in this genetic background (compare the cumulative disease index between control C57BL/6 and SJL/J mice in Table I). It may be that the higher level of inflammation seen in the C57BL/6 background overrides the protective effect of sCrry. It is unknown at this time whether complement levels in the CNS of C57BL/6 mice are significantly higher, either from higher baseline production or differential regulation under inflammatory conditions (or both), than those found in SJL/J mice. If C57BL/6 mice do have higher complement levels in the CNS, the ability of sCrry to prevent complement-mediated inflammation may be function of quantitative limitations. This possibility is supported by previous studies in which cobra venom factor depletion of systemic complement was effective in protecting against clinically mild but not severe EAE (10, 11). Another possible explanation is that different genetic backgrounds allow the development of different forms of EAE that may be either fully or partially dependent on complement activation. However, it is worth noting that in both genetic backgrounds, wild-type mice showed evidence for C4 deposition in CNS lesions, suggesting that complement is activated in these two forms of EAE. Mice homozygous for the sCrry transgene may be useful in addressing these questions and are currently in development.

MS is now considered a heterogeneous disease which may utilize diverse pathogenic mechanisms leading to a unique clinical presentation for each patient. Despite the heterogeneity of MS, inflammation and tissue destruction remain common features of the disease. As such, our results suggest a therapeutic potential for inhibiting complement activation in the treatment of demyelinating disease. Similar strategies have been suggested for other inflammatory CNS diseases in which complement has been implicated.

### Table I. EAE clinical features in control and sCrry-transgenic mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Incidence</th>
<th>Onset in Days (range)</th>
<th>Maximum Score (SD)</th>
<th>CDF* (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL/6 × SJL and BL/6</td>
<td>14/14</td>
<td>11 (8–15)</td>
<td>4.0 (1)</td>
<td>63.4 (23.1)</td>
</tr>
<tr>
<td>SJL</td>
<td>5/6</td>
<td>23 (22–24)</td>
<td>3.0 (1.3)</td>
<td>19 (16)</td>
</tr>
<tr>
<td>5–9 sCrry</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL/6 × SJL and BL/6</td>
<td>14/14</td>
<td>16 (9–27)*</td>
<td>3.8 (0.5)</td>
<td>46.9 (20.1)</td>
</tr>
<tr>
<td>SJL</td>
<td>0 /8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5–7 sCrry</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL/6 × SJL and BL/6</td>
<td>3/3</td>
<td>16.3 (15–17)</td>
<td>3.0 (2.5)</td>
<td>45 (58)</td>
</tr>
<tr>
<td>SJL</td>
<td>0/8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*The mean cumulative disease index (CDF) was calculated from the sum of the daily clinical scores.

\( p < 0.01 \) vs control (Student’s t test).

### FIGURE 4.

Extent of cellular infiltration and demyelination in sCrry transgenic mice compared with wild-type control mice during EAE. Hematoxylin and eosin staining of representative spinal cord sections from wild-type mouse with a C57BL/6 MHC phenotype (A), 5-9 sCrry transgenic mouse with a C57BL/6 MHC phenotype (B), wild-type mouse with a SJL/J MHC phenotype (C), and 5-9 sCrry transgenic mouse with a SJL/J MHC phenotype (D). Magnification, \( \times 200 \). Toluidine blue staining of representative spinal cord sections from a wild-type mouse with an SJL/J MHC phenotype (E) and 5-9 sCrry transgenic mouse with the SJL/J MHC phenotype (F). Spinal cord sections were analyzed 5 wk after immunization. Magnification, \( \times 400 \).
including Alzheimer’s disease (9) and traumatic brain injury (26). sCrry transgenic mice represent a unique and powerful tool to investigate the role of complement in the pathogenesis of these and other CNS diseases.

Acknowledgments

We thank Dr. Carl Pinkert, and his staff from the University of Alabama, Birmingham (UAB) Transgenic Resource, for help with the generation of the sCrry transgenic mice. Dr. Yancey Gillespie (Department of Neurosurgery, UAB) for help in obtaining the murine CSF samples, and Ed Phillips (Department of Neurobiology, UAB) for help with the Epon fixation and toluidine blue staining. We also thank Prof. Jean-Yves Muller for his support.

References