ORIGINAL ARTICLE

The Innate Immune Response to Adjuvants Dictates the Adaptive Immune Response to Autoantigens

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Abstract

To elucidate the role of innate immunity in susceptibility to the animal model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), we induced EAE by immunization with spinal cord homogenate (SCH) plus complete Freund adjuvant or carbonyl iron in 3 inbred rat strains. Lewis are considered “susceptible,” PVG/c-Rt7a (PVG) as “semisusceptible,” and Brown Norway (BN) as “resistant” to EAE. Immunization with SCH-carbonyl iron resulted in clinical disease in all 3 strains, but the pathologic features of EAE in the resistant BN and the semi-susceptible PVG rats differed from those in the Lewis and PVG model of EAE induced with SCH-complete Freund adjuvant. In BN and PVG rats, there were numerous inflammatory lesions with prominent involvement of microglia and, to a lesser extent, perivascular macrophages. These data suggest that different levels of activation of the innate immune system by different adjuvants determine whether EAE will or will not develop. Accordingly, the widely accepted scale of susceptibility to EAE development (Lewis > PVG > BN) should be revised because it does not take into account the important contribution of the composition of the adjuvant to the quality and quantity of the innate immune response and, consequently, to the generation and extent of the pathogenic T-cell-mediated, that is, adaptive, autoimmune disease.

Key Words: Autoimmune, Experimental autoimmune encephalomyelitis, Innate immunity, Microglia, Multiple sclerosis, Nitric oxide

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). The etiology of MS is not understood, but many studies suggest that MS results from the interplay of multiple genes with largely unknown environmental factors (1, 2). In the widely studied animal model of MS, experimental autoimmune encephalomyelitis (EAE), the susceptibility of different strains of genetically identical mice and rats to the development of the disease by immunization with diverse autoantigens has been evaluated (3–5). From such studies, it has been suggested that several factors, including differences in the cytokine milieu or other inflammatory mediators, variations in the processes of generation of regulatory cells and/or effectors cells, and differences in the perpetuation of the immune response in the CNS, may explain the disparate propensities of various rodent strains to develop EAE (6–9). Insight into these complex processes may elucidate the immunopathogenesis of MS.

The different EAE susceptibilities exhibited by various strains of rats have been associated with particular gene regions. Among these, considerable attention has been drawn to the major histocompatibility complex (MHC) (5). Genetic analyses of back-cross generations between susceptible and resistant strains have shown that the presence of certain Class II MHC haplotypes seems to determine the degree of susceptibility to develop EAE (10–13). It is now apparent, however, that other non-MHC regions of the genome also make particular contributions to disease susceptibility. For example, the EAE-resistant phenotype of Brown Norway (BN) and PVG/c-Rt7a (PVG) rats is thought to stem from an elevated activity of the hypothalamic-pituitary axis compared with that of susceptible Lewis (Lew) rats (14–16). Alternatively, other immune mediators (e.g. nitric oxide [NO]) acting directly on the effector cells or indirectly on other mediators of the immune response such as cytokines and chemokines have been shown to play an important part in modulating immune mechanisms that lead to the generation of harmful autoimmune responses (17, 18).

In recent years, based on the paradigm that the degree of polarization to a TH1 versus a TH2 phenotype in response to immunization determines susceptibility to EAE, various strains of rats have been classified according to a scale ranging from “susceptible” to “resistant” to EAE development (19). Lewis rats have been regarded as susceptible on the basis of their capacity to mount a robust TH1 cell immune reaction in response to immunization with various neuroantigens (10). In contrast, owing to their high tendency to produce a biased TH2-like response after immunization, BN rats have been considered resistant to EAE (19–22). It is now apparent, however, that there is no clear-cut distinction between susceptibility and resistance to EAE. Thus, under special circumstances, TH12-like responses against autoantigens, rather than purely TH1 or TH2 responses, may be the cause of EAE.
than protecting or controlling the deleterious autoimmune reaction, may play an important part in the mechanisms leading to disease development with or without the cooperation of T<sub>H1</sub>-like responses (23). Moreover, the reputed EAE-resistant phenotype exhibited by BN rats can be overridden by the use of myelin oligodendrocyte glycoprotein as immunogen and by the substitution of carbonyl iron (CI) for complete Freund adjuvant (CFA) as adjuvant for immunization (11, 12, 24, 25).

In this study, we evaluated the susceptibility to EAE in several rat strains, including Lew, which is known as susceptible; PVG, which is considered semisusceptible; and BN, which is traditionally believed to be a resistant strain. We demonstrate that immunization with spinal cord homogenate (SCH) mixed with CI (SCH-CI) results in clinical and pathologic disease in the 3 rat strains. Moreover, the pathologic features of the disease that develops in response to immunization with SCH-CI in the previously considered resistant and semisusceptible strains differ from those observed in the classic rat EAE model induced with neuroantigen plus CFA. The analysis in BN and PVG rats reveals numerous inflammatory lesions with extensive involvement of activated microglia and, to a lesser extent, peripheral macrophages, as highlighted using reconstituted bone marrow PVG chimeras. Our data suggest that different levels of activation of the innate immune system achieved in these experiments by the use of different adjuvants determines whether EAE will or will not develop. These results not only highlight the important role that the innate immune system plays in the complex interplay leading to the generation of autoimmune responses in the CNS but also raise the need to reassess the potential applicability to the human disease of the numerous genetic linkage studies in which putative disease regulating genes are mapped.

MATERIALS AND METHODS

Animals

Lewis, female PVG, and BN rats were purchased from the Animal Breeding Establishment at the Australian National University (Canberra, ACT, Australia). PVG/c-Rt7b rats were purchased from the Animal Resources Centre, Canning Vale, Western Australia. All procedures were carried out according to the guidelines set by the Animal Ethics Committee of the Australian Health and Medical Research Council.

Irradiation Bone Marrow Chimeras

Female PVG rats were irradiated with 1,000 rad and given intravenously 8.6 × 10^5 bone marrow cells from the congenic PVG/c-Rt7b rats. The animals were kept in sterile individual cages and given sterile food and water with 1 mg/ml oxytetracycline. One month later, samples of peripheral blood monocytes were tested by fluorescence-activated cell sorter for CD45 isofoms using the monoclonal antibody NDS-58. Rats that showed 100% chimerism were immunized with SCH-CI.

Active EAE Induction and Scoring

The procedure was as previously published (25). Briefly, 40% rat SCH was either 1) emulsified with an equal volume of CFA (incomplete Freund adjuvant containing 4 mg/ml Mycobacterium butyricum), and a total of 100 μl SCH-CFA were injected in both hind footpads; or 2) mixed with CI (5% CI; Sigma-Aldrich, Castle Hill, Australia), and 50 μl SCH-CI was injected in 1 hind footpad. Clinical disease was graded as 0, normal; 1, flaccid distal half of tail; 2, flaccid tail; 3, ataxia; 4, hindlimb paralysis; or 5, hind and front limb paralysis.

Splenocyte Interferon-γ Production and Proliferation

Mononuclear splenocytes (5 × 10^6; after Ficoll gradient density = 1.077 g/ml) were activated with 1 to 5 μg/ml concanavalin A (conA) for 50 hours, and the amount of interferon (IFN)-γ in the culture supernatants was measured by ELISA (17). The proliferation was measured in parallel cultures with ^3H-Thy during the last 15 hours.

Reactive Nitrogen Intermediate Assay

Splenocytes (5 × 10^6; after erythrocyte lysis) were exposed to various concentrations of rat recombinant IFN-γ (Genzyme, Suffolk, UK) for 40 hours, and reactive nitrogen intermediates (RNIs) were measured in the culture supernatants. Peritoneal macrophages (5 × 10^5) were exposed to various concentrations of rat recombinant IFN-γ and lipopolysaccharide (Sigma-Aldrich) for 40 hours, and RNIs were measured in the culture supernatants. The sum of nitrate and nitrite concentrations was used as an indirect measure for the amount of NO being produced. The method is described in Reference 26. Briefly, the nitrate was measured colorimetrically after addition of Griess reagent. Nitrate was first converted to nitrite by nitrate reductase in the presence of nicotinamide adenine dinucleotide phosphate (reduced form; Boehringer Mannheim, Mannheim, Germany). The standard curves were with nitrate and nitrite ranging from 0 to 1,000 μmol/L.

Fluorescence-Activated Cell Sorter Analysis

Single-cell suspensions of spleen and lymph node cells were prepared, followed by lysis of red blood cells in 0.83% NH<sub>4</sub>Cl. Cells were first treated with staining medium (PBS with 5% fetal calf serum and 0.05% NaN<sub>3</sub>). Antibodies used included phycoerythrin- or fluorescein isothiocyanate-conjugated anti-CD4 (OX-38), anti-CD8 (OX-8), anti-α,β-TCR (R-73), anti-CD25 (NDS-61), anti-CD45RC (OX-22), anti-CD80 (3H5), and anti-CD86 (OX-46) from Serotec (Oxford, UK); and anti-CD11b (OX-42) and anti-CD44 (OX-50) from Chemicon (Temecula, CA). Cells (1 × 10^6) were stained with 1 μg primary monoclonal antibody for 30 minutes on ice and washed twice with PBS. Controls (antibody isotype controls) were run in parallel. The fluorescence was determined using a flow cytometer (Beckman Coulter, Fullerton, CA), and analyses on gated lymphoid cells were performed using CellQuest (Becton Dickinson) software. The fluorescence-activated cell sorter analysis showed that all CD11b cells were ED1⁺.

Arginase Assay

The assay was performed as outlined by Corraliza et al (27). Briefly, frozen tissue was homogenized in lysis
buffer (0.1% Triton X-100, 2% complete protease inhibitor [EDTA-free tablet], Roche Diagnostics, Indianapolis, IN). To each lysate supernatant was added arginine activation solution (10 mmol/L MnCl₂ in 50 mmol/L Tris, pH 7.5) and 20 mmol/L arginine solution in 0.1 mol/L carbonate buffer (pH 9.7). The reaction was stopped with H₂SO₄, H₃PO₄, and water at 1:3.7 vol/vol, and ethanol solution of 96% 1-phenyl-1,2-propanedione-2-oxide (27) was added. After incubation for 45 minutes at 95°C, the insoluble material was removed by centrifugation. Aliquots from each sample were transferred into 96-well flat-bottom plates, and the outer diameter at 540 nm was measured in a microplate reader (ThermoMAX; Molecular Devices, Sunnyvale, CA).

**Immunohistochemistry**

After perfusion with cold PBS and 4% paraformaldehyde, the tissues were fixed in 4% paraformaldehyde. Immunohistochemistry was performed on routinely processed paraffin sections after antigen retrieval in citrate buffer (pH 6.0) for 20 minutes. The antibodies used included mouse anti-rat ED1 antibody and anti-rat CD8 (Serotec); rabbit anti-inducible nitric oxide synthase (iNOS) polyclonal antiserum (Sapphire Bioscence, Crow’s Nest, Australia). The staining procedure was according to the manufacturer instructions (Innogenex, San Ramon, CA), with aminophenyl carbazole as peroxidase substrate. A ligation (ISOL) apoptosis detection kit (Chemicon) was used on paraffin sections to identify apoptosis. Briefly, 5-μm paraffin sections were incubated with 3% of H₂O₂ in PBS to quench endogenous peroxidase activity. Antigen retrieval was performed by boiling sections in citrate buffer (pH 6.0) for 30 minutes. Sections were incubated with ApopTag equilibration buffer (Chemicon) for 1 minute at room temperature, followed by a mixture of T4 DNA ligase and a blunt-ended biotinylated oligo (Chemicon) for 18 hours at 4°C. Sections were further exposed to streptavidin-peroxidase for 1 hour at room temperature, and the positive reaction was visualized with diaminobenzidine as substrate (Chemicon). When double staining was necessary, 1 of the secondary antibodies was labeled with alkaline phosphatase, and Vector Blue alkaline phosphatase substrate kit III (Vector Laboratories, Burlingame, CA) was used as a substrate. When using PVG bone marrow chimeras, the immunohistochemical reactions were performed on cryostat sections of lumbar spinal cord using anti-ED1-biotin (Serotec) and NDS58-biotin antibodies (a kind gift by Dr Alex Bishop, Centenary Institute of Cancer Medicine and Cell Biology, Sydney, Australia).

Peritoneal macrophages from naïve Lew, PVG, and BN rats were collected with cold Hanks containing 5 U/ml heparin and left overnight in 8-well chambers in Dulbecco’s modified Eagle’s medium-10% FCS medium. The non-adherent cells were removed, and the adherent cells were exposed to rat recombinant IFN-γ ranging from 0 to 200 U/ml for 48 hours. The cells were fixed with paraformaldehyde, and immunocytochemistry for ED-1 was performed.

**Real-Time Polymerase Chain Reaction**

Rats were perfused with cold PBS, spleens were removed, and single-cell suspensions were prepared, followed by erythrocyte lysis in 0.83% NH₄Cl. Total RNA was isolated using Trizol technology (GIBCO, Invitrogen, Victoria, Australia). Purity of the RNA was determined by A₂₆₀/A₂₈₀. One microgram of RNA for each sample was reverse transcribed into cDNA using an Omniscript reverse transcription kit (Qiagen, Victoria, Australia).

SYBR-Green-based real-time polymerase chain reaction (PCR) was used to measure relative gene expression of indoleamine 2,3-dioxygenase (IDO) in each sample. Each master mix (20 μL) contained a single gene-specific primer set (sense and antisense, 2.5 mmol/L), 20 ng of cDNA, and 2XSYBR-Green PCR master mix (Applied Biosystems, Foster City, CA). Each experimental sample was assayed using 3 replicates for each primer, including the β-actin-specific primer that was used as an internal standard. Negative controls lacking the cDNA template were run with every assay to assess specificity. Primer Express software (Applied Biosystems) was used for primer design. Gene-specific primer sets were designed to span intron-exon junctions to discriminate between cDNA and genomic DNA. The primer sets used in these studies were as follows: β-actin, sense 5'-CGT GAA AAG ATG ACC CAG AT CA-3'; antisense 5'-CAC AGC CTG GAT GCC TAC GT-3'; IDO sense 5'-GGA GAA GAC CAA GGA AAT TT TAA-3'; antisense 5'-TGC GGA GAA CTT GGA AAA AC-3'. Polymerase chain reaction amplification was performed in a 7300 sequence detection PCR system (Applied Biosystems). The cycling conditions were as follows: 1 cycle at 95°C for 10 minutes, followed by 40 cycles of PCR amplification, each consisting of 95°C for 15 seconds and 60°C for 45 seconds. Sequence Detection software (SDS v 1.2.2; Applied Biosystems) was used for analysis of the results. A threshold cycle was determined for each sample. Polymerase chain reaction assays showing nonspecific products at the end point were excluded from further data analysis. Relative quantification using the comparative threshold cycle method (28) was used for analysis. The results were expressed as relative fold change over the values for untreated mice.

**Statistical Analysis**

Data were expressed as a mean ± standard deviation. Statistical analysis was performed using the GraphPrism program package (version 4; GraphPad Software, San Diego, CA). Data were evaluated by performing the Student unpaired, 2-tailed t-test or 2-way analysis of variance and Bonferroni tests as appropriate. A value of p < 0.05 was considered to be statistically significant.

**RESULTS**

**Immunization with SCH and CI as an Adjuvant Results in Enhanced Disease Development in Lew, PVG, and BN Rats**

It has been previously shown that BN rats, which are considered resistant to EAE induction after immunization with SCH-CFA, develop acute EAE when CI is used as adjuvant (24, 25). Here, we show that the increased EAE susceptibility of BN in response to SCH-CI is not an isolated
phenomenon because PVG rats, which are considered semi-susceptible, also showed increased incidence and severity of EAE after SCH-CI immunization (Table). Thus, whereas only 50% of PVG rats were susceptible to EAE induction with SCH-CFA, the incidence was nearly 100% when CI was used as the adjuvant. Moreover, the PVG rats immunized with SCH-CI had earlier onset of clinical disease, higher maximal clinical score, and longer disease duration compared with the SCH-CFA group. Finally, Lew rats immunized with SCH-CI also exhibited higher maximal clinical scores and longer mean episode duration than SCH-CFA-immunized Lew rats (Table).

Proliferation and IFN-γ, NO, and IDO Production by Lymphoid Cells Differ Among the 3 Strains of Rats After Activation

Interferon γ is a T_{H}1-like cytokine that has been widely implicated in the pathogenesis of EAE and MS (29). Accordingly, we next assessed the levels of IFN-γ secretion...
and the proliferation of splenocytes from naïve BN, PVG, and Lew rats in response to different doses of the polyclonal stimulator conA. Figure 1A shows that, at lower doses of conA, spleen cells from PVG and BN rats responded with a higher secretion of IFN-γ. On the other hand, at doses higher than 4 μg/ml, the pattern reversed, and spleen cells from Lew rats exhibited more pronounced secretion of IFN-γ secretion was examined together with the proliferation rate in response to conA, although splenocytes from PVG and BN rats secreted higher levels of IFN-γ at lower doses of the polyclonal activator, under these circumstances, the same cells were unable to sustain a significant in vitro proliferative response to conA (Fig. 1A). In Lew rats, spleen cells responded with a gradual elevation in proliferation, peaking at 3 μg/ml of conA, followed by a sharp reduction in proliferation at doses of 4 and 5 μg/ml. At higher doses of conA, the reduction in proliferation coincided with the interval of the IFN-γ secretion curve where the slope increased sharply.

We next evaluated the ability of mononuclear spleen cells isolated from the rat strains to produce regulatory immune mediators in response to IFN-γ. Figure 1B shows the levels of RNI (a measure of NO production) secreted by Lew, PVG, and BN splenocytes. At doses of IFN-γ from 2.5 to 25 U/ml, the BN splenocyte cultures produced significantly more RNI than PVG and Lew splenocyte cultures.

Two enzymes, arginase and NOS, are involved in arginine metabolism (30). Because it has been previously reported that an increased NO production could be a result of decreased arginase activity (30), we evaluated whether different levels of arginase activity are responsible for the disparate NO production between the rat strains. Accordingly, the arginase activity was measured in spleen and liver samples from 5 Lew and 5 BN rats on Day 10 after immunization with SCH-CFA. There was no difference in the values (microgram urea per milliliter) for the liver samples (1.713 ± 34 for Lew and 1.672 ± 73 for BN) or for the spleen samples (41.2 ± 12.7 for Lew and 62.9 ± 15.4 for BN). The iNOS seems to represent the major source of NO during inflammatory conditions (17). Because iNOS is mainly expressed in monocytes/macrophages, we next isolated macrophages from the peritoneal cavity of PVG and Lew rats and measured the release of RNI in response to several doses of IFN-γ and lipopolysaccharide. As shown in Figure 1B, regardless of the doses of IFN-γ and lipopolysaccharide, BN and PVG rat macrophages responded with consistently higher levels of RNI compared with those of Lew rats.

Indoleamine 2,3-dioxygenase is an enzyme, induced preferentially by IFN-γ, which catalyzes the initial and rate-limiting step in the degradation of the essential amino acid tryptophan (31, 32). It has been suggested that IFN-γ activation of IDO plays a critical part in the regulation of the immune response. Because splenocytes from BN and PVG rats responded with higher levels of IFN-γ, we assessed by real-time PCR the expression level of IDO in the BN, PVG, and Lew in response to activation. Figure 1C shows that after activation by polyclonal activator, splenocytes isolated from PVG and BN rats express higher levels of IDO compared with Lew rats. Taken together, these results suggest that macrophages from PVG and BN rats have lower activation thresholds compared with macrophages from Lew rats.

**Lysosomal Activation of Macrophages From Different Rat Strains**

To confirm the suggestion raised by the previous experiments, we assessed the proportion of ED-1⁺ cells after stimulation with different doses of IFN-γ. ED-1 is a lysosomal marker, and its upregulation indicates cell activation. At concentrations of IFN-γ from 2.5 to 10 U/ml, the BN and PVG peritoneal macrophages responded with a gradual increase in ED-1 expression detected by immunohistochemistry (Supplementary Fig. 1). In contrast, peritoneal macrophages from Lew rats did not upregulate ED-1 expression in response to these IFN-γ doses. Once again, they were less sensitive to IFN-γ with respect to ED-1 upregulation; doses greater than 75 U/ml IFN-γ were required to induce ED-1. Therefore, these data further support the conclusion that the threshold of activation in response to in vitro exposure to IFN-γ varies considerably in the different rat strains. Such differences may explain, in part, the distinctive responses of the innate immune system of each rat strain after immunization.

We have previously shown that the innate immune system of BN rats responds aggressively to the amalgam of compounds that constitute CFA. As a result of this higher responsiveness, when CFA is used as adjuvant for EAE induction, the BN rats produce copious amounts of NO in the spleen during the early stages of the immune response, as determined by immunohistochemical staining for iNOS (25). Accordingly, we next evaluated the response of the innate immune system of all 3 strains of rats after immunization with SCH-CFA and SCH-CI by assessing ED-1⁺ cells in the spleens. As shown in Supplementary Figure 2A, PVG and BN rats reacted to immunization with SCH-CFA with an extensive increase of ED-1 positivity in the spleen. In sharp contrast, when CI was used as adjuvant for immunization, only moderate numbers of ED-1⁺ cells were observed in all rat strains. It should be mentioned that the PVG rats seemed to show higher ED-1 positivity compared with Lew rats in response also to SCH-CI immunization. However, the nature of the method used for this assessment does not allow us to draw a definitive conclusion. We also examined the livers from the 3 rat strains and observed more ED-1 positivity in the livers of BN and PVG rats than in the Lew rat livers after immunization with CFA (Supplementary Fig. 2B). None of the rat strains exhibited significantly enhanced ED-1 expression in the liver when CI was used as adjuvant.

**Cell Phenotypes in the Popliteal Lymph Nodes and Spleen After Immunization of Rat Strains With SCH-CFA or SCH-CI**

To understand the mechanisms underlying the generation of EAE effector cells after immunization with SCH-CFA or SCH-CI, the cellular composition and the phenotype of the lymphoid lymph node and spleen cells were analyzed by flow cytometry. Apart from consistent low numbers of CD8 T cells in the BN rat compared with Lew and PVG rats (as has been previously reported by others [19], we did not
find significant differences between the rat strains in the numbers of other T cells [α,β-TCR-CD4+ and CD8+ cells] or B cells in both immune organs and at the time points analyzed [data not shown]). In contrast, when the monocyte compartment was analyzed, BN and PVG rats had significantly higher numbers of CD11b+ cells in their lymph nodes in response to SCH-CI compared with SCH-CFA on Days 3 and 6 after immunization (Fig. 2A). This difference was not seen in the Lew rats because this strain showed virtually the same numbers of CD11b+ cells after immunization with either adjuvant. When the cellular composition of the spleen was assessed on Day 6, higher numbers of CD11b+ cells were observed in BN and PVG rats after immunization with SCH-CFA compared with SCH-CI (Fig. 2A). The number of CD11b+ cells in the Lew rat spleen was the same regardless of the adjuvant used for immunization.

For activation of autoreactive T cells, antigen-presenting cells (APCs) present antigenic peptides on their MHC Class II molecules and provide costimulatory signals through CD80 and CD86 to their counterreceptor CD28 on T cells (33). Therefore, we analyzed the levels of expression of CD80 and CD86 in Lew, PVG, and BN after immunization with SCH-CFA or SCH-CI. As shown in Figure 2B, 3 days after immunization, the levels of expression of CD80 and CD86 in lymph node CD11b+ cells were higher in the groups of PVG and BN rats that had received CI as adjuvant compared with those that had received CFA. In sharp contrast, CI and CFA induced comparable levels of expression of costimulatory molecules in Lew rats. These data highlight differences in antigen presentation to autoreactive T cells in the periphery in the different rat strains.

**Histopathologic Examination of the CNS Infiltrate in Lew, PVG, and BN Rats After Induction of EAE by SCH-CFA or SCH-CI**

CNS inflammatory cell infiltration during EAE was examined in the lumbar spinal cords of Lew, PVG, and BN rats that had clinical scores of 3.5 after immunization with carbonyl iron as an adjuvant resulted in experimental autoimmune encephalomyelitis (EAE) with numerous ED-1+ cells in the lumbar spinal cord parenchyma. Inducible nitric oxide synthase (iNOS)-positive cells appeared at recovery from clinical disease. The panels for iNOS (at EAE score 3.5 and recovery from EAE scores 3.5 to 2) are from sections adjacent to those for ED-1. Brown Norway rats not showing signs of disease were killed at different points, and no inflammatory lesions were found in any sample.
SCH-CFA or SCH-CI. After immunization with SCH-CFA, Lew and PVG rats had typical meningeal and perivascular inflammation, whereas BN rats did not display any CNS abnormalities (Fig. 3; Row 5). In Lew rats, there were few ED-1+ cells in perivascular cuffs (Row 1), whereas in the PVG rats, many ED-1+ cells were in both perivascular inflammatory cuffs and in the parenchyma (Row 3). In sharp contrast, all rats immunized with SCH-CI had inflammatory lesions in the lumbar spinal cord. No differences were observed in the extent and location of the mononuclear cell infiltration or the numbers of ED-1+ cells between SCH-CFA- and SCH-CI-immunized Lew rats (Rows 1 and 2), but BN (Row 6) and PVG rats (Row 4) reacted strongly to SCH-CI immunization, and both strains displayed numerous inflammatory lesions containing large numbers of perivascular and parenchymal ED-1+ cells.

The iNOS expression in the lumbar spinal cord after immunization with SCH-CFA and SCH-CI was also examined by immunohistochemistry. As shown in Figure 3 (Column 3), iNOS+ cells were seldom identified early in disease but became apparent at peak disease.

We next performed immunohistochemistry on the lumbar spinal cord of rats that had recovered from EAE. Figure 3 shows that during recovery from EAE, the ED-1 positivity decreased in the spinal cord in all rat strains regardless of the adjuvant used for the induction of disease. Despite the decrease in the number of ED-1+ cells, an increase in iNOS immunostaining was consistently observed at recovery. This latter observation suggests a role for iNOS as a key regulator during CNS inflammation.

In EAE, the CNS macrophage populations are derived from 2 distinct populations of cells, that is, monocyte/macrophages from the periphery and resident microglia (34). It is, however, extremely difficult to distinguish between the 2 cell types during CNS inflammation. To determine whether the large numbers of ED-1+ cells in the lumbar spinal cord of PVG rats in response to SCH-CI immunization are derived from macrophages or parenchymal microglia, we induced EAE in bone marrow chimera rats using CI as adjuvant. Irradiated PVG rats were reconstituted with bone marrow cells from congenic PVG/c-Rt7b rats and then immunized with SCH-CI. As shown in Figure 4A, after immunization with SCH-CI, ED-1+ cells were distributed in perivascular spaces and in the parenchyma of the lumbar spinal cord of the chimeric rats. Immunolabeling with the NDS58 antibody indicated that cells from the donor (NDS58+ cells), that is, the cells that had migrated from the blood, were localized close to the blood vessels (Fig. 4B), rather than in the parenchyma. To further confirm that ED-1+ cells in the parenchyma were microglial cells, peripheral phagocytic cells from PVG rats were labeled by triple (on Days 5, 7, and 9 postimmunization) intravenous injection of India ink. The analysis revealed that ink-labeled cells were only located around the blood vessels (Fig. 4C). Figure 4D shows the total absence of ink-labeled cells in the parenchyma.

**FIGURE 4.** ED-1+ cells distributed throughout the CNS parenchyma are mainly parenchymal microglia. PVG/c-Rt7a rats were reconstituted with bone marrow cells from congenic PVG/c-Rt7b rats and then immunized with spinal cord homogenate (SCH)-carbonyl iron (CI). When clinical experimental autoimmune encephalomyelitis (EAE) signs were present, lumbar spinal cords were collected. (A) Staining with anti-ED-1 antibody shows that the ED-1+ cells (red) are located in perivascular and parenchymal areas. (B) A serial section from the lesion shown in (A) stained with NDS-58 antibody (red) reveals that the blood-derived inflammatory cells are mainly located around the blood vessels. (C, D) Peripheral phagocytic cells were labeled with India ink. (C) Green arrows point to ink-positive cells of an EAE perivascular lesion after SCH-CI immunization. (D) No ink-phagocytic cells were observed among the ED-1+ cells in the CNS parenchyma.

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At recovery from EAE, the staining for ED-1 in the perivascular and spinal cord parenchymal cells of PVG and BN rats became weak and patchy (Figs. 5A, B). We asked whether this reflects only decreased activation of ED-1+ cells or whether it is accompanied by cell death. Apoptosis has previously been suggested as one of the mechanisms underlying the elimination of activated macrophages and microglia in the recovery phase of the disease (35–37). Double immunocytochemical staining for ED-1 showed that the cells undergoing apoptosis were always around parenchymal blood vessels and in the meninges and were either ED-1 negative or showed patchy ED-1 staining (Figs. 5C, D). On the other hand, whereas ED-1+ cells located in the parenchyma also showed patchy ED-1 staining during recovery from EAE, not many of these cells were identified as apoptotic (Figs. 5E, F). These observations suggest that in SCH-CI-induced EAE in PVG and BN rats, the infiltrating lymphocytes and monocytes induce activation in many glial cells, but that at recovery from an EAE episode, blood-derived inflammatory cells undergo apoptosis, whereas most of the parenchymal ED-1+ cells revert to a non-activated stage.

DISCUSSION

Experimental autoimmune encephalomyelitis is traditionally induced in rodents using a combination of CFA and neuroantigen. After such protocols, different strains of mice and rats are classified as susceptible or resistant on the basis of their ability to develop the neuroinflammation that leads to CNS dysfunction and clinical signs (19, 38, 39). A multitude of genetic linkage studies have been performed to elucidate
EAE susceptibility and resistance, and several genes or variants of genes have been shown to influence parameters such as the generation of autoreactive cells and the disease course. These studies have resulted in the identification of several regions in the genome of various rat strains that affect susceptibility or resistance to EAE (5, 13–16, 40, 41).

We show here that the susceptibility of various rat strains strongly depends on the protocol used for immunization insofar as it affects the innate immune response. Thus, we found that with the use of CFA as adjuvant, the Lew rat is susceptible, the PVG is semisusceptible, and the BN is resistant to EAE. On the other hand, when CI is used as adjuvant, all 3 strains are highly susceptible. Our results seem to indicate that despite the T-cell-mediated nature of EAE, the collaboration of the innate immune system is an essential regulating force for its development. Our study also emphasizes the exceptional qualities of CI as adjuvant for EAE development. Finally, our experiments show that in PVG and BN, immunization with SCH-CI lead to a disease characterized by a massive activation of resident microglial cells. Together with the results of in vitro experiments, the latter observation provides a comprehensive framework from which the rat strains used can be ranked. There is an inverse relationship between the innate immune response and the adaptive immune response that results in clinical EAE. Therefore, for the innate response, BN > PVG > LEW, whereas for the adaptive response, LEW > PVG > BN.

From a mechanistic point of view, we and others have shown that the development of a T-lymphocyte-dependent autoimmune process such as EAE is strongly influenced by regulatory molecules secreted by macrophages, that is, NO and IDO, which affect the generation and expansion of T effector cells and/or their transendothelial migratory ability (18, 31, 32). Our present results show that in response to lower doses of the polyclonal activator conA, immune cells from BN and PVG rats secreted higher amounts of IFN-γ, which is the major activator of both iNOS (i.e., the classical pathway) and IDO (17, 42). Moreover, when equivalent doses of IFN-γ were added to cultures, splenocytes from PVG, and particularly BN rats, released higher amounts of IDO and NO compared with Lew rats. Together with the dynamics of ED-1 expression by macrophage cultures from 3 rat strains (BN, PVG, and Lew), this clearly indicates that lower inflammatory stimulant is required for triggering the activation of PVG and especially BN macrophages compared with Lew macrophages.

We have previously shown that when female PVG rats are treated with an iNOS inhibitor (N-methyl-L-arginine), they develop a fulminant disease in response to immunization with neuroantigen emulsified with CFA. These results seemed to stem from limiting the effect of NO on T₁eff cell proliferation (43). Following on these results, therefore, we postulate that the elevated levels of NO secreted by BN rats are in part responsible for the lack of development of clinical signs or histologic EAE in response to SCH-CFA. We have, however, been unable to revert the resistant phenotype exhibited by BN by treating them with N-methyl-L-arginine. This may reflect either that the toxicologic profile of N-methyl-L-arginine does not allow to achieve the pharmacologic effect required for inhibiting iNOS or that other regulatory molecules also account for limiting the immune response after immunization with CFA.

During the last 50 years, CFA has represented an irreplaceable tool for the induction of EAE (44). The use of CFA is believed to enhance the specific immune response directed to the neuroantigen added to the emulsion by prolonging the presence of the antigen in the injection site and therefore facilitating persistent antigen stimulation (45). It is now known that one of the attributes of CFA is to induce the expression of B7 costimulatory molecules on APCs as a result of the recognition of bacterial components by pathogen-associated molecular pattern receptors expressed on the surface of several immune cells (44). In the present study, despite lack of bacterial components in the mixture of SCH-CI, CD11b⁺ cells in the lymph nodes from PVG and BN rats responded to immunization with a significant elevation in the expression of B7 molecules. Moreover, in PVG and BN rats, the upregulation of B7 molecules on the surface of APCs in response to SCH-CI was higher than when CFA was used as adjuvant for EAE induction. On the other hand, in Lew rats, the levels of B7 expression were practically indistinguishable in response to the 2 adjuvants. Together, this indicates that the interaction of APCs with iron particles in the absence of bacterial components is an extremely efficient way to promote the generation of EAE effector cells in BN and PVG rats. Furthermore, the presence of bacterial components in the immunizing mixture is not required for the induction of EAE in susceptible strains such as Lew because they are also highly susceptible to EAE induced by immunization with SCH-CI.

Immunization with CFA resulted in a marked increase in the number of monocytes/macrophages in spleens and livers of PVG and BN rats. This increase, however, begins to be apparent on Day 6 after immunization, in contrast to the earlier changes observed in the number and expression of costimulatory molecules in the lymph nodes of the 3 rat strains. One possible interpretation of this result is that the spleen plays a very important role in the regulation of the generation of effector cells during the later stages of the development of the autoimmune response. In support of this, the elevated number of CD11b⁺ cells in the spleens of BN and PVG rats is consistent with the high systemic values of RNI in these EAE-resistant rat strains in response to CFA immunization (22, 25, 26).

A histologic study of the CNS in PVG and BN rats after immunization with SCH-CI showed high numbers of ED-1⁺ cells distributed through the parenchyma. On the other hand, in Lew rats, both immunization protocols (SCH-CFA and SCH-CI) resulted in similar neuroinflammatory findings, that is, lymphocytes and moderate numbers of ED-1⁺ cells that were usually limited to perivascular inflammatory cell cuffs. It is generally believed that peripheral macrophages and resident microglia directly contribute to CNS damage in MS and EAE by several mechanisms, including the production of proinflammatory cytokines, matrix metalloproteinases, and free radicals (46). Accordingly, the severe histologic disease in the BN and PVG rats can be ascribed to pathologic mechanisms that result from the marked activation of
macrophages and/or microglia in the CNS in response to SCH-C1 immunization.

Our finding that most of the ED-1+ cells in the parenchyma were resident microglia also seems to reflect the low threshold of activation of the innate immune system in BN and PVG rats. Indeed, other authors have previously found that ramified microglia from BN and PVG rats express higher constitutive levels of MCH Class II molecules compared with Lew rats (47, 48). However, based on the resistant and semiresistant phenotype exhibited by BN and PVG rats in response to CFA, those authors concluded that there was an inverse relationship between the constitutive expression of MHC Class II complexes on the microglia and the susceptibility to CNS autoimmunity (48). They implicitly assumed a scale of susceptibility of EAE development on the basis of the previously reported resistant and semisusceptible phenotypes of BN and PVG, respectively. In view of our results, it seems that such a scale of susceptibility is only valid when CFA is used as adjuvant for immunization. Moreover, our observations seem to lead to the opposite assumption, in which the higher constitutive expression of MHC Class II complexes on microglia by BN rats might be directly correlated with their elevated susceptibility to CNS autoimmunity.

In conclusion, this study highlights the crucial role that the adjuvant used for immunization plays in defining the susceptibility to EAE development. The type of adjuvant seems to be responsible for the level of activation of the innate immune response, which may in turn inhibit the adaptive response.

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