Hippocampal Damage in Mouse and Human Forms of Systemic Autoimmune Disease

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Abstract

Systemic lupus erythematosus (SLE) is frequently accompanied by neuropsychiatric (NP) and cognitive deficits of unknown etiology. By using autoimmune MRL-lpr mice as an animal model of NP-SLE, we examine the relationship between autoimmunity, hippocampal damage, and behavioral dysfunction. Fluoro Jade B (FJB) staining and anti-ubiquitin (anti-Ub) immunocytochemistry were used to assess neuronal damage in young (asymptomatic) and aged (diseased) mice, while spontaneous alternation behavior (SAB) was used to estimate the severity of hippocampal dysfunction. The causal relationship between autoimmunity and neuropathology was tested by prolonged administration of the immunosuppressive drug cyclophosphamide (CY). In comparison to congenic MRL +/+ controls, SAB acquisition rates and performance in the "reversal" trial were impaired in diseased MRL-lpr mice, suggesting limited use of the spatial learning strategy. FJB-positive neurons and anti-Ub particles were frequent in the CA3 region. Conversely, CY treatment attenuated the SAB deficit and overall FJB staining. Similarly to mouse brain, the hippocampus from a patient who died from NP-SLE showed reduced neuronal density in the CA3 region and dentate gyrus, as well as increased FJB positivity in these regions. Gliosis and neuronal loss were observed in the gray matter, and T lymphocytes and stromal calcifications were common in the choroid plexus. Taken together, these results suggest that systemic autoimmunity induces significant hippocampal damage, which may underlie affective and cognitive deficits in NP-SLE.

Keywords

autoimmunity; inflammation; lupus; hippocampus; Fluoro Jade B; ubiquitin; spontaneous alternation behavior; cyclophosphamide; MRL mice

INTRODUCTION

Cognitive and affective disorders are frequent manifestations of the systemic autoimmune/inflammatory disease lupus erythematosus (SLE). Largely due to multi-system involvement and confounding factors (e.g., uremia and treatment with corticosteroids), clinical and experimental studies have thus far not succeeded in elucidating the principal factors and

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mechanisms involved in the pathogenesis of neuropsychiatric (NP)-SLE (Bruyn, 1995). With an ultimate goal of understanding the central nervous system (CNS) targets and functional consequences of SLE-like disease, we have been examining the neuropathology and behavioral dysfunction in the MRL-MpJ-Tnfrsf6[lpr] (MRL-lpr) mouse substrain. This substrain is known to develop systemic autoimmune disease due to a *lpr* mutation on chromosome 19 and a dysfunctional Fas (CD95, APO-1) receptor in a negative selection of autoreactive T cells (Watanabe-Fukunaga et al., 1992a,b). Along with early onset of inflammation and autoimmunity, MRL-lpr mice display changes in emotional reactivity (Sakic et al., 1994b), deficits in spatial learning/memory tasks (Sakic et al., 1992, 1993; Hess et al., 1993), accumulation of serotonin in the hippocampus (Sakic et al., 2002), and atrophy of pyramidal neurons (Sakic et al., 1998). Consistent with a case report on isolated hippocampal damage (Schnider et al., 1995) and impaired cognitive function in SLE patients (Carbotte et al., 1995; Denburg et al., 1997; Hanly et al., 1999), these results suggested structural and functional damage of the hippocampus during systemic autoimmunity and inflammation.

Behavioral deficits in MRL-lpr mice are defined as the departure from the behavioral performance of congenic MRL-MpJ +/+ (MRL +/+) controls (Szechtman et al., 1997), which develop similar disease symptoms later in life (Theofilopoulos, 1992). Marked behavioral deficits in the MRL-lpr substrain have been observed in mice as early as 7 weeks of age (Sakic et al., 1994a) and coincide with the emergence of humoral autoimmunity, but antedate generalized lymphadenopathy, arthritis, glomerulo-nephritis, and skin lesions (Andrews et al., 1978). The initial support for the hypothesis that spatial learning and memory are impaired by lupus-like disease came from studies in which MRL-lpr mice perseverated in their response bias during extinction and reversal learning in the Morris water-maze task (Sakic et al., 1992, 1993). The aim of the present study is to evaluate the time course of spontaneous alternation behavior (SAB), a functional trait proposed to be highly sensitive to hippocampal damage (reviewed in Richman et al., 1986). More specifically, it is well documented that spatial working memory and the reliable alternation of rodents in a T-maze in two consecutive trials largely depend on an intact hippocampus (Lalonde, 2002). If development of autoimmunity induces hippocampal damage, one might expect that in comparison to congenic MRL +/+ controls, diseased MRL-lpr mice would show a reduced SAB rate at advanced stages of lupus-like disease, but not before the serological signs of autoimmunity are manifest.

Although neuronal degeneration in the CA2/CA3 regions of MRL-lpr brains was recently demonstrated with a novel Fluoro Jade B (FJB) cytochemical stain (Ballok et al., 2003), it has not been determined whether this damage is functionally important. In addition, it remains to be determined whether neuronal loss is associated with the progress of autoimmune disease or, alternatively, reflects a developmental deficiency related to impaired expression of the apoptotic Fas receptor in the MRL-lpr brain (Park et al., 1998). Although the latter possibility appears less viable in the light of evidence that cortical architectures in young, pre-diseased MRL-lpr and MRL +/+ mice are comparable (Sherman et al., 1990), and that size of hippocampal fields and neuronal density are not reduced in Fas-deficient *lpr* mice (Kovac et al., 2002), we assess age-related changes in densities of hippocampal neurons stained by the hematoxylin-eosin (H&E) method. The present analysis involves the comparison between young (asymptomatic) and old (diseased) MRL-lpr and MRL +/+ mice and employs ubiquitination as an additional marker of cell degeneration (Alves-Rodrigues et al., 1998). Specifically, ubiquitin (Ub) binds to damaged or misfolded proteins, targeting them for degradation by the Ub-proteasome pathway. If ubiquitinated proteins are not eliminated by this pathway, neurodegeneration may occur. In addition to cellular demise, ubiquitination of proteins may be involved in a DNA repair mechanism (Jentsch et al., 1987). Therefore, it was expected that an increased density of FJB-positive cells and alterations in the Ub-proteasome degradation system in the hippocampus would parallel both the emergence of autoimmunity and impaired SAB performance. To test for a cause-effect relationship between neuronal

degeneration and autoimmunity, the immunosuppressive drug cyclophosphamide (CY) was used as previously reported (Farrell et al., 1997; Sakic et al., 1995, 2000a).

Despite the well-acknowledged construct validity and theoretical usefulness of animal models, it is equally important to demonstrate their face validity (Henn and McKinney, 1987). Our experimental study coincided with the death of an SLE patient who had CNS involvement. This provided us with a unique opportunity to compare neuropathological changes in animal and human forms of systemic autoimmune disease in which brain function is compromised.

MATERIALS AND METHODS

Experiment I: Age-Related Changes in SAB Response Rate and Neuromorphology

Animals—Three-week-old (±3 days) MRL-lpr and MRL +/+ male mice (n = 20 mice/ substrain) were purchased from the Jackson Laboratory (Bar Harbor, ME); each group was left for 7 days to habituate to local laboratory conditions (light phase: 8 AM–8 PM, food, and water ad libitum; 5 mice/cage; level E, cages sanitized during regular housing). Five days prior to behavioral testing, mice were singly caged and habituated to the experimenter (Sakic et al., 1992). In addition, mouse cages were wheeled on three occasions from the colony room to the testing room, to reduce stress induced by transportation and a novel environment. Ten mice from each strain were tested and sacrificed at either 6 or 16 weeks of age (cohort 1). Because of dissimilar fixation protocols for the assessment of dying neurons and ubiquinated particles, a second batch of mice (cohort 2; 20 males/substrain) was obtained from our recently established MRL colony (housing level A, cages sterilized, replaced, and manipulated under laminar flow protection). At 3 weeks of age (±3 days), the mice were transferred to a housing room and left to habituate over 7 days, for assessment of brain morphology at 4 and 14 weeks of age. All experimental protocols were performed in accordance with the rules and regulations of the Canadian Council of Animal Care.

Spontaneous alternation behavior—There is substantial evidence that the hippocampus is one of the structures most intimately involved in SAB (Lalonde, 2002). Indirect but compelling evidence that supports this notion comes from developmental studies in which hippocampal maturation and level of SAB show parallel courses (Deacon et al., 2002). Considering that hippocampal damage reduces the rate of SAB in mice (Deacon et al., 2002), it was expected that young MRL-lpr mice would show a comparable SAB rate to congenic controls before the onset of autoimmunity and, conversely, that their SAB rate would decline with advancing manifestations of systemic autoimmune disease.

The T-maze (made of black Plexiglas) consisted of four perpendicular arms (H = 15 × L = 25 × W = 10 cm) and sliding separators that could modify the maze into an L, T, or + shape. The discrete-trial procedure (Richman et al., 1986) was employed, with a daily session consisting of trial 1 (5 s in a start position and entry into an unblocked arm), 60-s intertrial period (mouse restrained in the arm) and trial 2, in which both arms were open and a mouse was expected to choose an unvisited arm after leaving the start position. An arm was considered to have been chosen if all four limbs were within the selected arm. A guillotine door was lowered behind the mouse, immediately after the entry. Urinary trails were removed, and the maze was cleaned by a cloth moistened with Windex® after each mouse was tested. The sequence of blocked arms (left or right) in trial 1 was randomly generated by Microsoft Excel software. Following 5 days of SAB testing, mice were given a reversal trial on day 6, to examine whether the non-spatial learning strategy was employed (Bertholet and Crusio, 1991), such as taxis or praxis (Sutherland and McDonald, 1990; Whishaw, 1991). Trial 1 consisted of placing a mouse in the usual starting position. However, following a 60-s intertrial period, trial 2 was initiated from the arm located 180 degrees from the trial 1 arm. Assuming that a mouse had used a

spatial strategy to memorize external cues, it was expected that after leaving the new start position, an unvisited arm would be chosen by a body turn identical to trial 1.

Indices of autoimmunity—High levels of serum antinuclear antibodies (ANA) and splenomegaly are typical manifestations of systemic autoimmune lupus-like disease (Theofilopoulos, 1992) and were presently examined. Mice were anesthetized with Somnotol (i.p. 60 mg/kg body weight; MTC Pharmaceuticals, Cambridge, ON) and perfused with 40 ml of phosphate-buffered saline (PBS) after terminal bleeding from the vena cava. Blood samples were left to coagulate in 1.5-ml plastic vials and centrifuged for 10 min at 3,000 rpm. Serum was separated from the clot and stored at −20°C until further analysis. ANA concentration was measured using a sandwich Anti-Nuclear Antibody ELISA kit (Cat. no. 5200), according to the manufacturer's instructions (Alpha Diagnostic International, San Antonio, TX) and the protocol previously described (Sakic et al., 2000a). In brief, serum samples were diluted 1:100 in the kit diluent and applied to both experimental and control wells to assess the specificity of binding. Optical density was determined using a microplate ELISA reader set to 450 nm. The wet spleen weight was determined on an analytical scale (Sartorius 2024 MP, VWR Scientific of Canada Ltd.) shortly after extraction.

Fluoro Jade B method—The Fluoro-Jade B stain has an affinity for the entire degenerating neuron, regardless of the type of cell death (Hopkins et al., 2000; Schmued and Hopkins, 2000). Despite incomplete knowledge of the staining mechanisms, the FJB method shows high reliability in the detection of dying neurons (Ye et al., 2001).

Extracted brains were fixed in 4% paraformaldehyde (PFA) for 24 h and were then immersed in 30% sucrose (in PBS) for 4 days before frozen sections were processed according to the previously published protocol (Ballok et al., 2003). In brief, slides were immersed in 100% ethanol for 3 min, followed by 1 min in 70% ethanol. The slides were rinsed in distilled water (dH_2O) for 1 min before being transferred to a 0.06% potassium permanganate solution and gently shaken for 15 min. Slides were rinsed in dH_2O for 1 min before immersion in a 0.001% FJB/0.1% acetic acid staining solution (prepared from a 0.01% stock solution; HistoChem, Jefferson, AR). In our experience, the stock solution produced optimal staining results after 2 months of storage at 4°C in darkness. After 30 min of gentle shaking in the staining solution, slides were rinsed for 1 min in each of three dH_2O washes and left to dry for several hours in darkness. Subsequently, they were processed in three 2-min xylene washes before being coverslipped with DPX (Sigma, St. Louis, MO). The reactivity in the CA3 sector and nonhippocampal periventricular areas were examined using an epifluorescent microscope with 450 – 490-nm excitation light (Diastar Fluorescence Microscope, Reichert Scientific, Buffalo, NY) at ×200 magnification. To quantify the number of FJB-positive neurons, sections were photographed on 400 ASA 35-mm print film (Eastman Kodak, Rochester, NY), using a Nikon N90s camera. The pictures were scanned at 600-dpi resolution and digitized into TIFF files using Adobe Photoshop software (Adobe Systems, San Jose, CA) for assessment by NIH Image analysis software (Scion, Frederick, MD). To produce representative high-quality images, a Zeiss Laser Scanning Confocal Microscope (LSM 510, Carl Zeiss) argon laser (wavelength 488 nm) was employed for visualization of FJB. Confocal micrographs were obtained using a Fluar 20×/0.75 objective in combination with a 1,024 × 1,024-pixel resolution and were saved in the TIFF format.

The overall neuronal density was assessed by H&E staining of coronal sections (bregma approximately −1.0 mm) in the parietal cortex and CA2/CA3 area that were less densely packed with neurons and thus amenable to quantification. Sections were stained with an automated slide stainer (Leica Instruments, Germany), and 1-mm^2 areas were examined with a stage micrometer by an unbiased observer. Sections of the highest quality were included in the

analysis (n = 4 – 8 brains/group) and four counts (2 sections × 2 hemispheres) were performed at ×400 magnification.

Immunohistochemical localization of Ub—Mice were perfused with PBS, and the brains were extracted within 2 min, immersed into 10% neutral buffered formalin, and left in formalin for 4 days at room temperature (RT) until processing. Subsequent to fixation, brains were embedded in paraffin and cut in the coronal plane at 4 μm. Serial adjacent sections were stained with H&E and processed immunohistochemically for Ub, using an anti-Ub polyclonal antiserum (1:400; Dako, Burlington, Canada) and the labeled streptavidin-biotin-peroxidase technique (Vectastain; Shandon, Pittsburgh, PA). Sections were briefly counterstained with hematoxylin, dehydrated in a graded series of ethanol, mounted in xylene, and coverslipped. For each animal, the number of Ub-immunopositive dot-like structures was counted in five nonoverlapping fields within the strata oriens and pyramidale of the CA3 sector of the hippocampus. All counts were performed at the same coronal level (bregma −1.6 to −2.4 mm) under oil immersion (×1,000) by an experimenter blind to the experimental design.

Experiment II: Effects of Immunosuppression on SAB and Neuromorphology

Animals—To examine whether prolonged immunosuppressive treatment modifies SAB performance, 24 MRL-lpr and 20 MRL +/+ male mice (3 weeks old ±3 days) were purchased from the Jackson Laboratory. An additional batch of 12 MRL-lpr and 12 MRL +/+ mice was purchased to confirm behavioral observations and examine the effects of sustained immunosuppression on neuropathology seen in Experiment I. Each group was managed and maintained under conditions as outlined above. Two weeks later (i.e., 5 weeks of age), mice were housed singly to receive treatment.

Immunosuppressive treatment—The therapeutic effect of CY on the development of autoimmune symptoms was demonstrated previously (Shiraki et al., 1984; Grota et al., 1989, 1990; Sakic et al., 1995, 1996). In addition to the reduction of leukocyte numbers (Snippe et al., 1976), CY makes these cells unresponsive to stimuli, leading to generalized immunosuppression (ten Berge et al., 1982). CY was injected weekly (100 mg/kg i.p.; mouse LD_{50} = 405 mg/kg i.p.; Procytox, Horner, Montreal, Canada) to half of the mice in each group. The treatment started during the 5th week of life and ended during the14th week. The other half of the animals received nine injections of an equivalent volume (~0.2– 0.3 ml) of saline (SAL). Mice were assigned into one of four groups, according to substrain (MRL-lpr vs MRL +/+) and treatment (CY vs SAL). In both batches, two mice died prematurely in the CY group before treatment was completed. To avoid acute effects of CY and stress induced by injection, the SAB testing commenced 7 days after the last injection and mice were sacrificed at around 16 weeks of age. FJB and Ub staining was analyzed as described above, and all counts were performed by an observer blind to the experimental design. ANA levels, which correlate highly with the spleen weight (Sakic et al., 2000a), were assessed as in Experiment 1.

Experiment III: Human Specimens

Brain tissue was obtained from a 58-year-old woman who died from NP-SLE and had a history of psychosis and seizures. She presented with status epilepticus that was managed with phenytoin and diazepam. She remained comatose after her seizures. Despite aggressive immunosuppression, including high-dose methylpred-nisolone, plasmapheresis, and CY, her condition deteriorated; she developed disseminated intravascular coagulation and died. The brain was examined after 14-day fixation in 10% buffered formalin. Sections were taken from the meninges, blood vessels, pons, medulla oblongata, cerebellum, mamillary bodies, right and left hippocampus, basal ganglia, frontal watershed area, temporal, parietal and occipital lobes, periventricular area around lateral and third ventricles, and choroid plexus. Coronal sections of the brain were serially sectioned and representative paraffin wax embedded tissue sections

were stained with Luxol fast blue/H&E. Other stains used were Bielschowsky silver and Congo red. Using standard immunohistochemical methods, sections of the periventricular area, hippocampus, and temporal gray matter were stained for Ub; sections of the choroid plexus were stained for leukocyte common antigen (LCA), T-cell markers (CD3, CD4, CD8), B-cell marker (CD20), and macrophage marker (CD68). All sections were examined under light microscopy. Additional sections were frozen, stained with FJB, and examined with confocal microscopy. Control sections were obtained from a female subject of similar age who died of causes unrelated to SLE and epilepsy.

Statistical Analysis—The data were analyzed by analysis of variance (ANOVA) with substrain (MRL-lpr vs MRL +/+), treatment (CY vs SAL), and age as between-group factors, and slide as the repeated measure. Student's t-test was used in the post hoc analysis. Fisher's exact test was used to assess the difference in group performance in the SAB tests. Significance level was set at $P < 0.05$, and all computations were performed using the SPSS 11.0 statistical package. Graphs show means ± SEM.

RESULTS

Experiment I

The SAB performance in young mice from the two MRL substrains was comparable at 6 weeks of age. However, 16-week-old MRL-lpr mice performed poorly in comparison to age-matched MRL+/+ controls ($t_{18} = 2.228$, $P = 0.039$, Table 1). This deficit was confirmed in the "reversal" trial, where the alternation rate of diseased MRL-lpr mice dropped to chance levels (Fisher's exact test, one-tailed $P = 0.016$, Table 1). Neuropathological assessment revealed higher numbers of FJB-positive neurons (Fig. 1A) in the CA3 region (strain by age: $F(1,36) = 39.944$, $P < 0.001$, MRL-lpr vs age-matched MRL +/+, $t_{18} = 6.583$, $P < 0.001$, Fig. 2A). In a separate cohort of 14-week-old MRL-lpr mice, immunostaining for Ub showed intensely immunoreactive spherical dot-like structures (Fig. 1C), which were numerous in the neuropil of the stratum oriens and superficial stratum pyramidale of the CA3 region (strain $F(1,18) = 8.093$, $P < 0.011$, $t_{18} = 3.000$, $P < 0.008$; Fig. 2A). In addition to the hippocampus, the substantia nigra and brainstem tegmentum also showed more Ub particles than in MRL +/+ controls (strain $F(1,18) = 8.998$, $P < 0.008$, $t_{18} = 2.845$, $P < 0.011$; data not shown). Examination at higher magnification showed that the morphology of the structures was reminiscent of axon terminals. Indeed, occasional linear arrays of these structures indicated the presence of immunoreactive varicose axonal segments.

Reduced density of cortical neurons in diseased MRL-lpr mice has been initially revealed with the cresyl violet method, but this observation was not quantified (Sakic et al., 1998). We presently confirmed this by assessing neuronal densities in the parietal cortex and CA2/CA3 region on sections stained with the standard H&E method. Comparisons across ages and to age-matched controls showed a paucity of cells in older MRL-lpr mice (Table 2). Despite a relatively small sample in some groups (n = 4 brains), the counts obtained suggest that significant group differences at an older age is a combination of an age-related increase in neuronal density in the MRL +/+ substrain and genuine loss of neurons during the development of systemic autoimmunity in the MRL-lpr substrain. This is supported by comparable immune statuses at younger ages, and the emergence of the SAB deficit when spleen weight and serum ANA titers increased in aged MRL-lpr mice (at 16 weeks of age, strain by age: for spleen, $F(1,36) = 22.448$, $P < 0.001$; for ANA, $F(1,35) = 28.110$, $P < 0.001$; at 14 weeks of age, strain by age: for ANA, $F(1,34) = 15.006$, $P < 0.001$; for spleen, $F(1,36) = 36.568$, $P < 0.001$; Fig. 2B,C).

Experiment II

The SAB deficit was less severe in CY-treated MRL-lpr mice compared to the SAL-treated MRL-lpr group ($t_{22} = 2.691$, $P = 0.013$; Table 2). CY-treated MRL-lpr mice also did not differ in the "reversal" test when compared to other groups (Table 3). Although the acquisition of SAB response in the CY-MRL +/+ group was comparable to that in the SAL-MRL +/+ group, their performance in the "reversal" task dropped to a chance level. This detrimental effect of CY on performance of control mice is not presently clear, but it is consistent with our previous observations (Sakic et al., 1995,1996). Our recent study demonstrated that sustained CY treatment normalizes neuronal morphology, as evidenced by Golgi impregnation (Sakic et al., 2000a). In the present study, extensive FJB staining appeared to be attenuated after immunosuppressive treatment. However, likely due to the small sample size (n = 5–6 mice/group), a trend was seen in the CA3 region (strain by treatment: $F(1,17) = 3.956$, $P = 0.063$; Fig. 3A) and a statistically significant difference was detected in other periventricular gray matter regions (strain by treatment: $F(3,17) = 5.549$, $P = 0.008$; between SAL and CY MRL-lpr groups, $t_9 = 2.161$, $P = 0.05$; Fig. 3B). This reduction in cytochemical staining was associated with the profound immunosuppressive effect of CY, as indicated by low ANA levels in the CY groups across two batches (batch: $F(1,56) = 0.022$, n.s.; strain by treatment: $F(1, 56) = 47.376$, $P < 0.001$; CY-lpr mice: 0.28 ± 0.06; SAL-lpr mice: 1.58 ± 0.14; CY +/+ mice: 0.13 ± 0.02; SAL +/+ mice: 0.24 ± 0.05). However, analysis of the CA3 region with the present sample size could not detect a beneficial effect of CY on the density of Ub-positive particles. Namely, sustained immunosuppression did not appear to change the difference between the MRL substrains (strain: $t_{19} = 3.006$, $P = 0.007$; treatment: $t_{19} = 0.157$, n.s.).

Experiment III

The brain of the lupus patient weighed 1,145 g. There was no evidence of a subdural hematoma, and both hemispheres were symmetrical with no evidence of meningeal exudates or herniations. Blood vessels, dissected out from the circle of Willis, did not show any thromboembolic or atherosclerotic changes, but they did show myointimal proliferation with some hyalinization of smaller blood vessels (features suggestive of a history of benign hypertension). On serial sectioning, there was no evidence of infarction or of intracerebral or intraventricular bleeding. There was dilation of the lateral and third ventricles, left more than right, with thinning of the cortex around the ventricles. Some of the intraparenchymal blood vessels showed perivascular cuffing by chronic inflammatory cells and deposition of hemosiderin, features suggesting previously healed vasculitis. This is further corroborated by the fact that there were foci of demyelination around these vessels, indicating a vasculitic mechanism probably related to immune complex deposition. There was no microscopic evidence of abnormal neuronal migration, infarction, intraparenchymal, or intraventricular hemorrhage. Conversely, gross examination of the control brain did not show ventricular dilation of the lateral and third ventricles.

An interesting feature was the presence of increased densities of scattered inflammatory cells within the choroid plexus of the lupus patient (Fig. 4A). Mononuclear lymphocytes displayed positive immunostaining for LCA, CD3, CD4, and CD8, indicating that these were T lymphocytes. More CD8 than CD4 cells were observed, suggesting that most of the T lymphocytes were cytotoxic (Fig. 4C). These were not present in the control brain (Fig. 4D). Furthermore, periventricular tissue around the lateral and third ventricles showed loss of neurons, gliosis, satellitosis (microglial cells surrounding occasional viable neurons), and "brain sands"/corpora amylacea, not present in the control (Fig. 5A–D). The parietotemporal cortex also showed gliosis and ischemic changes in the neurons (likely secondary to status epilepticus). The neuronal loss in the CA3 region of the hippocampus was accompanied by surviving neurons, gliosis, and focal satellitosis (Fig. 6A). This was also seen above the dentate gyrus in the pyramidal layer, in addition to patchy areas of cell loss (Fig. 6C). In contrast, the

hippocampus sections from the control brain did not show neuronal loss, gliosis, or satellitosis in the CA3 region. There were no patchy areas of cell loss in the pyramidal layer above the dentate gyrus. Neuronal demise in the hippocampus of the NP-SLE patient was confirmed by FJB staining. More specifically, FJB-positive neurons (Fig. 6E) and few scattered Ub-stained particles were observed in the CA3 and dentate gyrus. However, with respect to Ub staining, no significant difference between lupus and control brains could be detected.

DISCUSSION

The emergence of FJB-positive neurons and Ub-positive structures in the hippocampus of mice with impaired SAB and the beneficial effects of immunosuppressive treatment point to the causal link between chronic autoimmunity/inflammation, structural damage, and aberrant behavior. These findings complement documented atrophy of basilar and apical dendritic branches in the CA1 region and parietal cortex (Sakic et al., 1998, 2000a) and neurotoxic properties of cerebrospinal fluid (CSF) from diseased MRL-lpr mice (Maric et al., 2001). In comparison to the murine form of the disease (Denenberg et al., 1992; Farrell et al., 1997; Sakic et al., 2000a,b), similar changes are seen in the brain from the NP-SLE patient. They include neuronal loss in the hippocampus and parietal regions, T-lymphocyte infiltration into the choroid plexus, and ventricular enlargement. Overall, the present results further support our hypothesis that systemic autoimmune disease induces brain damage and subsequently, behavioral dysfunction. In addition, the above evidence strengthens the face and construct validity of MRL-lpr mice as a model of NP-SLE.

The SAB paradigm is a procedure proposed to reflect exploratory behavior, which also depends on the formation of working memory (Richman et al., 1986). However, anxiety and stress may negatively affect SAB (Bats et al., 2001) to the extent that it does not reflect exploratory behavior (Gerlai, 2001). In our study, the animals were initially habituated to the experimenter and the testing environment to reduce these confounding effects. In addition to consistently poorer performance over successive trials, a profound deficiency in the "reversal" trial was observed in aged MRL-lpr mice. If the spatial strategy was employed, MRL-lpr mice would be expected to alternate to the novel arm by making the same body turn on the second trial. However, the MRL-lpr mice used the opposite body turn to enter the unvisited arm, thus ending up in the same arm as in trial 1. This observation suggests that during the acquisition phase (across-the-day SAB rate in MRL+/+ mice increased from 70% to 90%, in MRL-lpr mice from 40% to 80%) the diseased mice did not rely on an extramaze spatial map to the same extent as MRL +/+ controls. Detrimental effects of prolonged CY treatment on performance of control MRL +/+ mice in the "reversal" trial is consistent with its negative effects in paradigms that measure exploration and motivated behavior (Sakic et al., 1995, 1996). Although the principal mechanisms are unclear, one may hypothesize that by cross-linking strands of DNA/RNA and inhibiting protein synthesis, CY treatment impairs molecular mechanisms (e.g., phosphorylation) required for short-term memory formation and consolidation (Ng et al., 1991). Over the past 40 years, lesion studies demonstrating reduced SAB rate in rodents have implicated the role of the hippocampus in attention control and memory formation (Roberts et al., 1962). If so, increased degeneration in the CA3 region of MRL-lpr brains may account for deficits in formation of a spatial map. However, more recent evidence suggests that in addition to hippocampal damage, the SAB deficit may reflect severed connections with other brain regions vital for the formation of working memory (Lalonde, 2002).

The loss of hippocampal neurons may occur via several non-mutually exclusive mechanisms. First, a direct pathway may involve increased permeability of the blood-brain barrier and infiltration of circulating immune factors into the CNS. The choroid plexus appears to be the primary site of immune complex deposition (Lampert and Oldstone, 1973; Vogelweid et al., 1991), which facilitates increased entry of soluble immune factors, monocytes and

lymphocytes into the parenchyma (Hess et al., 1993; Farrell et al., 1997) and hippocampal regions of lupus-prone mice (Kier, 1990). Subsequent leukocyte clustering may lead to the dissemination of autoreactive clones into the CSF (Sakic et al., 2000b). Whether systemically or intrathecally, these lymphocytes could produce neuroactive cytokines, chemokines, and/or brain-reactive antibodies (Hoffman and Madsen, 1990; Khin and Hoffman, 1993; Crimando and Hoffman, 1992, 1995; Hickey et al., 1997; Zameer and Hoffman, 2001), which compromise the survival of differentiated neurons (Maric et al., 2001). Indeed, when injected into the mouse hippocampus, the CSF from a demented NP-SLE patient induced apoptotic neuronal death via cross-reactive binding of anti-DNA antibodies to an N-methyl-D-aspartate (NMDA) receptor subtype (DeGiorgio et al., 2001). Given that neuronal survival is dependent on the activity of supporting cells, activation of microglia during the autoimmune disease (Hickey et al., 1997) may also lead to the generation of potentially neurotoxic factors (e.g., reactive oxygen species) which contribute to neuronal demise (Wood, 1998).

A second possibility is that glucococorticoids contribute to the loss of hippocampal neurons via cytokine-induced sustained activation of the hypothalamo-pituitary-adrenal (HPA) axis (Wick et al., 1993) and intracellular glucocorticoid receptors (McEwen et al., 1992). It is documented that glucocorticoids potentiate the release and postsynaptic actions of glutamate, which likely account for loss of neurons in the CA3 region (Magarinos et al., 1997). In vivo studies confirm that this region is vulnerable to endogenous glucocorticoids (Armanini et al., 1990) and that memory deficits and structural damage are prevented by an inhibitor of corticosterone synthesis (Roozendaal et al., 2001). Since MRL-lpr mice have chronically elevated basal levels of corticosterone (Hu et al., 1993; Lechner et al., 2000), this steroid may act in a similar fashion to induce neuronal loss in the CA3 region.

Pro-inflammatory cytokines are upregulated systemically (Tang et al., 1991; Tsai et al., 1995) and in the hippocampus of MRL-lpr mice (Tomita et al., 2001). Since they appear to alter central neurotransmission (Zalcman et al., 1994), one may hypothesize that an accumulation of serotonergic neurotoxins, such as 5,7-dihydroxytryptamine (Tabatabaie et al., 1993), could compromise the survival of hippocampal neurons. Indeed, we recently identified by HPLC excessive postmortem levels of serotonin (5-HT) in the hippocampus of MRL-lpr mice (Sakic et al., 2002). The observed increase in FJB and Ub staining in the CA3 region may reflect neuronal loss (due to an intracellular accumulation of toxic metabolites), which leads to impaired performance in tasks contingent upon the integrity of hippocampal circuits. However, more detailed assessment of the phenotype of dying neurons (e.g., serotonin transporter) combined with in situ analysis of central cytokines is required to confirm this relationship.

Highly Ub-immunoreactive structures were observed in brains from autoimmune MRL-lpr mice. They likely represent degenerating axon terminals, and their location suggests that they belong to mossy fibers. The damage observed in the hippocampal synapses in human neuropathological diseases (DeKosky and Scheff, 1990; Samuel et al., 1994) and aging (Gray et al., 2003) is proposed to have significant cognitive and behavioral consequences. Interestingly, antibodies reacting with Ub and ubiquitinated histones are present in SLE, with almost 80% of patients having antibodies against Ub-protein conjugates (Muller and Schwartz, 1995). However, the present treatment with CY neither reduced expression of Ub in brains from MRL-lpr mice nor did the analysis of the brain from a NP-SLE patient show increased density of Ub-positive particles. With respect to the animal model, it needs to be determined whether overexpression of Ub is associated with anti-brain reactivity, such as autoantibodies to Ub particles in kidneys (Elouaai et al., 1994), is a consequence of nonimmune pathogenic mechanisms (e.g., hormonal), or is merely an epiphenomenon of a systemic autoimmune disease.

The CNS and choroid plexus pathology in the MRL-lpr strain has been reported extensively (Alexander et al., 1983; Vogelweid et al., 1991; Hess et al., 1993; Farrell et al., 1997; Sakic et al., 2000b). Similarly, clinical reports point to the possibility that the choroid plexus is a site for immune complex and leukocyte deposition (Atkins et al., 1972; Gershwin et al., 1975; Peress et al., 1981; Duprez et al., 2001). In the present study, we provide a direct comparison of animal and human brain pathology by using the same protocols and reagents for FJB staining. In addition, the cortical thinning, dilation of ventricles, and infiltration of T lymphocytes into the stroma of the choroid plexus are comparable to ventricular enlargement (Denenberg et al., 1992) and the infiltration of cells immunoreactive for CD3, CD4, and CD8 antigens in the choroid plexus of MRL-lpr mice (Sakic et al., 2000b; Ballok et al., 2003). The neuropsychiatric manifestations reported in human SLE have also been shown to accompany cerebral atrophy (Chinn et al., 1997) and progressive neuronal loss (Sibbitt and Sibbitt, 1993; Brooks et al., 1997). In line with this evidence, the parietotemporal cortex of our patient showed gliosis and ischemic changes in neurons. Consistent with the notion of cortical damage, reduced neuronal density was presently observed by H&E in the parietal cortex of diseased MRL-lpr mice.

Schnider et al. (1995) described an SLE patient who presented with severe amnesia due to isolated hippocampal damage. Similar to this case report, the present analysis revealed neuronal loss in the CA3 region and the dentate gyrus, with surviving neurons in the pyramidal layer showing focal satellitosis and gliosis. Although CY treatment may have beneficial effects in preventing NP-SLE manifestations (Boumpas et al., 1991; Ramos et al., 1996), it neither prevented a fatal outcome in our patient nor showed effects comparable to those seen in lupus-prone mice. One explanation is that the dose of CY used in mice (100 mg/kg) is essentially myeloablative in humans and substantially higher then the standard treatment for human autoimmune diseases (1–2 mg/kg i.v. was given daily to our patient). Second, in experimental studies, effective immunosuppressive treatments are started well before overt signs of the disease (O'Sullivan et al., 1995; Sakic et al., 1995; Walker, 2001). This is similar to the clinical protocols in which CY treatment (often associated with side effects) needs to be employed early in order to prevent or minimize irreversible organ damage (Ioannou and Isenberg, 2002). However, the SLE patient in our study received the CY treatment when she became comatose (i.e., 4 days after she presented with status epilepticus), which was probably not sufficient to reduce existing brain damage.

In summary, the present results support the hypothesis that systemic autoimmune/inflammatory disease impairs hippocampal function and compromises neuronal survival in lupus-prone mice. Damage in the CA3 region is detectable after the spontaneous onset of the disease, affects performance in the SAB and "reversal" test, and can be attenuated by immunosuppressive treatment. Further studies are required to elucidate neuropathogenic mechanisms and neuronal phenotypes susceptible to the disease process.

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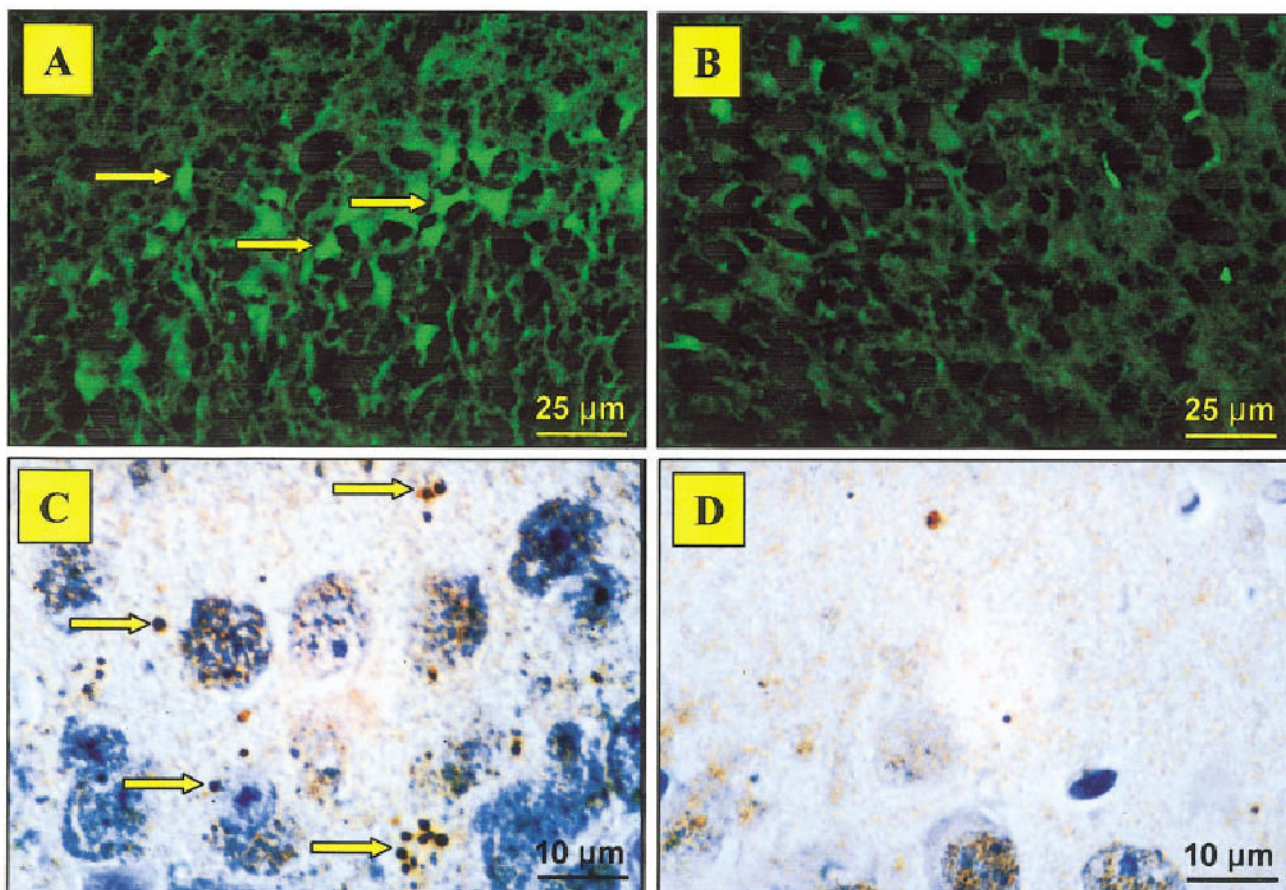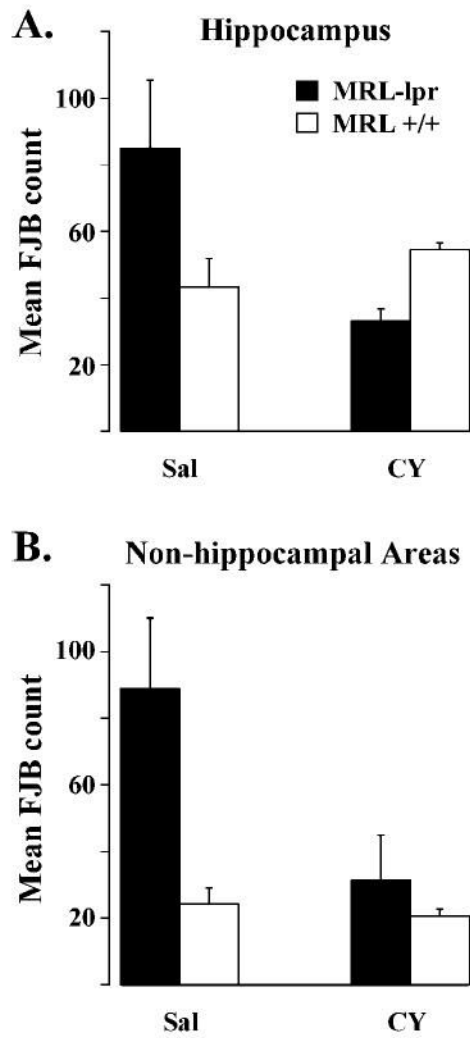
FIGURE 1.
Representative micrographs showing Fluoro Jade B (FJB) and ubiquitin (Ub) labeling in the CA3 region. Numerous FJB-positive cells with neuronal morphology (shown by arrows) were common in brains from MRL-lpr mice (A) in comparison to asymptomatic MRL +/+ controls (B). Intensely immunoreactive Ub-positive spherical particles (shown by arrows) were detected in brains from diseased lupus-prone mice (C), likely reflecting degenerating axon terminals. Such particles were not abundant in the control brains (D). ×400 in A,B; ×1,000 in C,D. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].
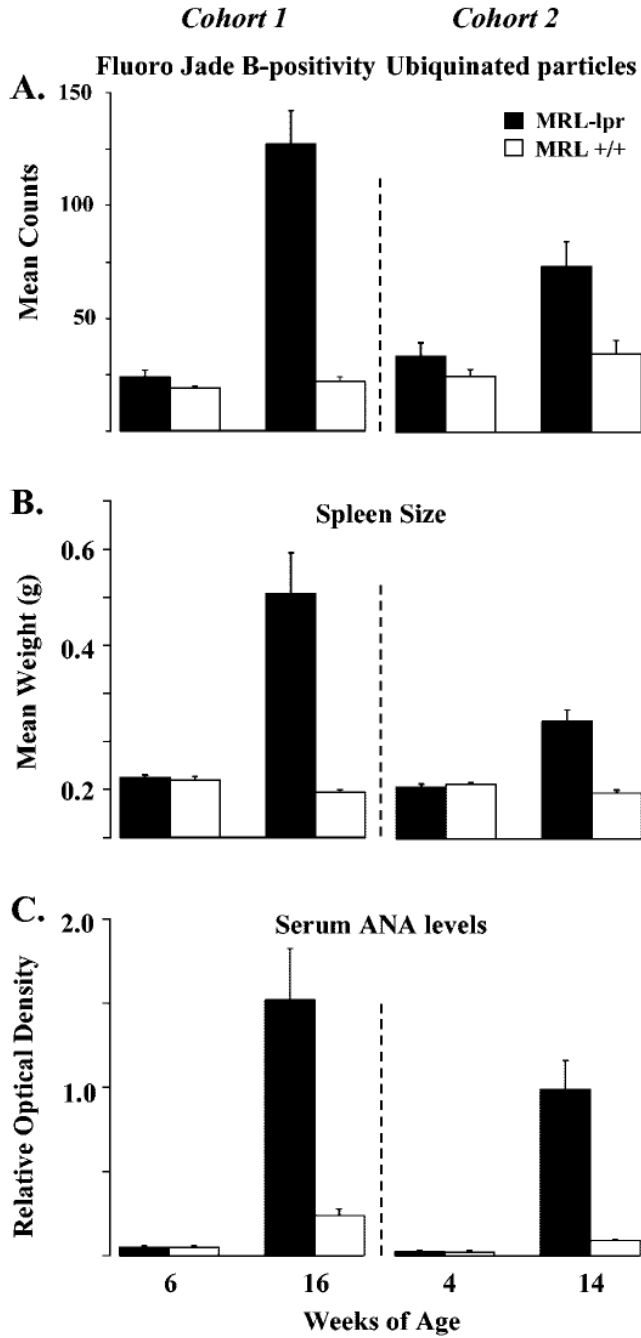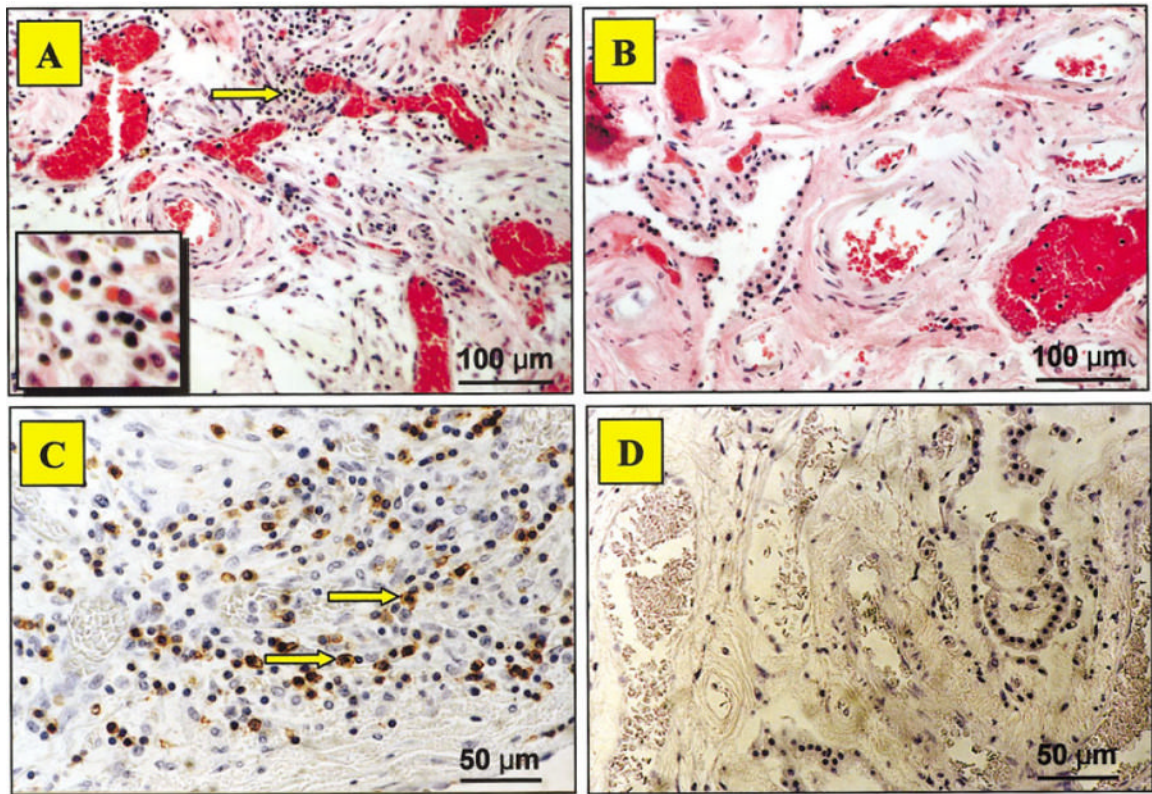
FIGURE 2.
Total numbers of Fluoro Jade B (FJB)-positive and ubiquitin (Ub)-positive cells and the immune status in MRL mice at different ages. Cells/particles staining for FJB or Ub significantly increased following the onset of autoimmunity in older MRL-lpr mice (A). Spleen weight (B) and antinuclear antibody (ANA) levels (C) also increased significantly with age, confirming the autoimmune status in the MRL-lpr substrain.

**A.** Hippocampus

B. Non-hippocampal Areas

FIGURE 3.
Effects of immunosuppressive treatment on brain pathology in MRL mice. Cyclophosphamide (CY) treatment appeared to attenuate the incidence of Fluoro Jade B (FJB)-positive cells in the hippocampus (A) and significantly reduced the staining in other brain regions of MRL-lpr mice (B).
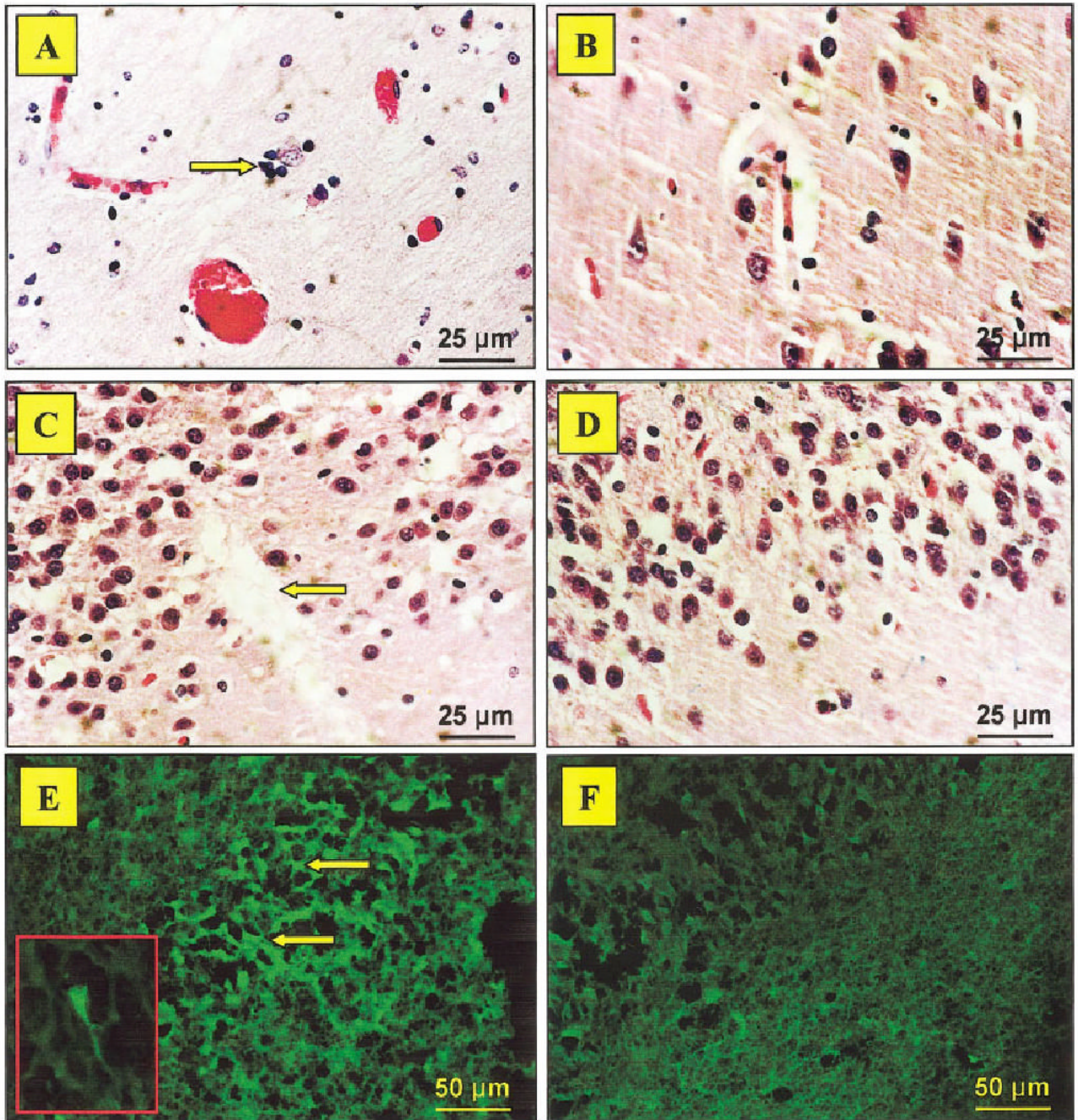
FIGURE 4.
Chronic mononuclear inflammatory cells in the human brain. H&E staining showed a cluster of lymphocytes (arrow) in the choroid plexus of the lupus patient (A), but not in the control brain (B). Subsequent immunohistochemical staining for leukocyte common antigen (LCA), CD3, CD4, and CD8 antigens (shown by arrows) confirmed that the cells in the stroma were T lymphocytes (C), while the control brain was negative for the same markers (D). ×100 in A,B; ×200 in C,D; ×600 (inset in A).
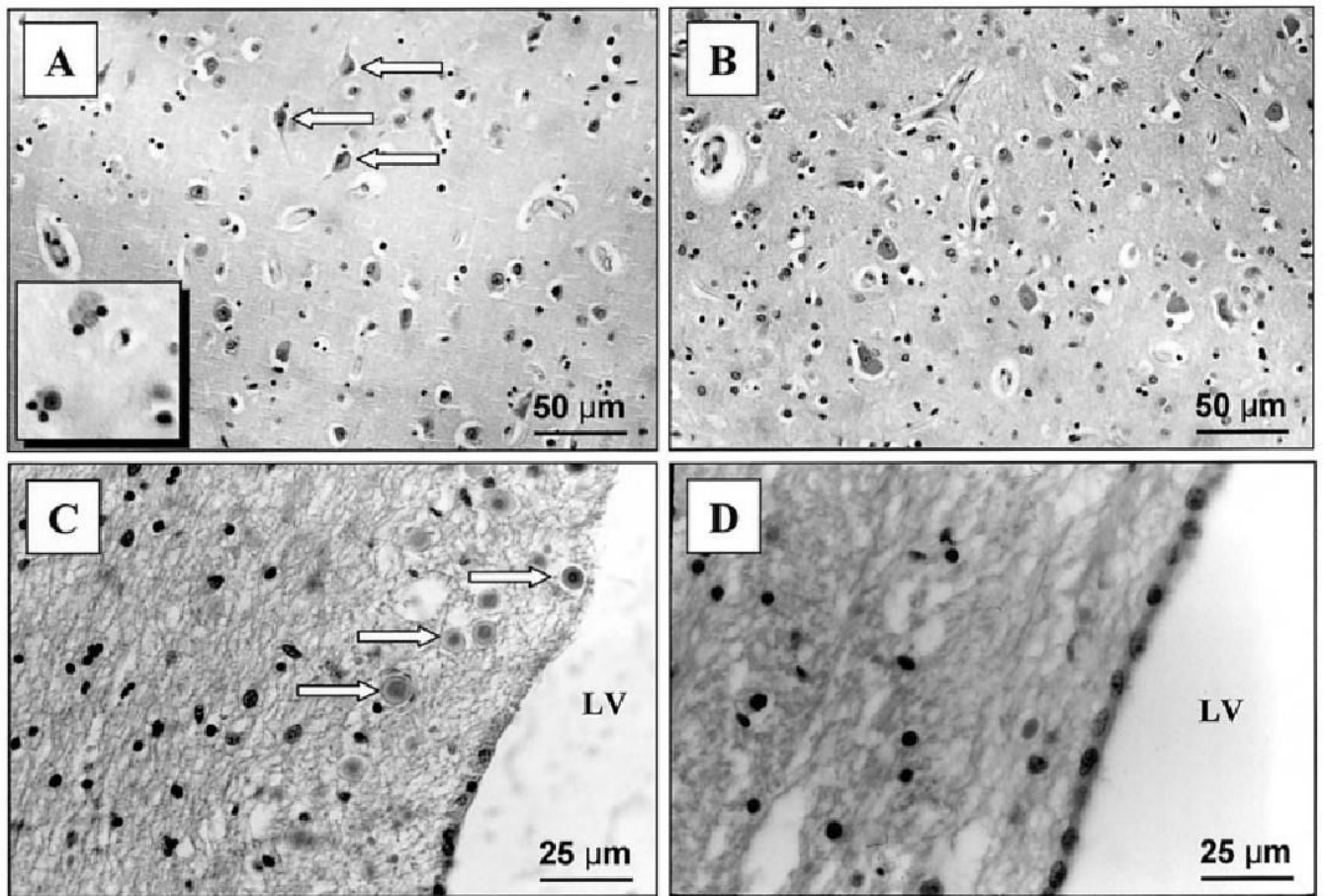
FIGURE 5.
H&E staining of the parietotemporal cortex and periventricular regions of the human brain. "Dying neurons" (shown by arrows) and satellitosis (Inset) in the gray matter of the lupus patient (A). Normal neurons and resting glial cells in the control brain (B). Numerous particles identified as "brain sand" were common around the ventricles in the patient's brain (C), and were rarely seen in the control (D). LV, lateral ventricle. ×200 in A,B); ×400 in C,D.

FIGURE 6.
Neuronal loss and degeneration in the human hippocampus as revealed by H&E and FJB staining. The brain from the neuropsychiatric-systemic lupus erythematosus (NP-SLE) patient revealed a paucity of neurons and satellitosis (arrow) in the CA3 region (A), while the control brain showed normal neuronal density (B). A patchy area of cell loss was also seen in the dentate gyrus (arrow) of the lupus brain (C), but not in the control (D). Subsequently, bright green Fluoro Jade B (FJB)-positive neurons confirmed a degenerative process in the CA3 region (E), which was not observed in the control brain (F). ×400 in A–D; ×200 in E,F; ×600 (inset in E, under oil immersion). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].

TABLE 1

SAB Rates (%) of Young (6-wk) and Older (16-wk) MRL-lpr and Control MRL +/+ Mice Over 5-Day Response Acquisition and Single Reversal Trial[†]

Group	Acquisition at 6 wk	Acquisition at 16 wk	Reversal at 16 wk
MRL-lpr (n = 10)	76 ± 5	64 ± 6[*]	50 (5/10)[**]
MRL +/+ (n = 10)	76 ± 5	80 ± 4	100 (10/10)

SAB, spontaneous alternation behavior.

[†] The alternation rate was lower in diseased 16-week-old MRL-lpr mice; a deficit in the spatial learning strategy was detected in the "reversal" task. This task differed from testing in the acquisition phase as Trial 2 was initiated 180 degrees away from the box arm where Trial 1 was given.

[*] In comparison to MRL+/+, $t_{18} = 2.228$, $P = 0.039$.

[**] In comparison with Fisher's exact test $P = 0.016$.

TABLE 2

Neuronal Density (Total Count ± SEM) as Assessed by Hematoxylin and Eosin Staining*[†]*

Group	Parietal cortex	CA2/CA3 area
6-wk MRL-lpr	514 ± 32 (n = 4)	210 ± 34 (n = 8)
6-wk MRL +/+	573 ± 32 (n = 7)	237 ± 33 (n = 7)
16-wk MRL-lpr	451 ± 43 (n = 8)[*]	159 ± 28 (n = 8)[**]
16-wk MRL +/+	691 ± 83 (n = 4)	250 ± 29 (n = 7)

[†] The total number of H&E-stained neurons was obtained from four 1-mm^2 areas (2 sections × 2 hemispheres) that were well preserved and amenable to counting (number of brains processed is shown in parentheses). Consistent with previous reports on reduced growth of brain mass and atrophy of pyramidal neurons, reduced neuronal density in diseased 16-week-old MRL-lpr mice supported the hypothesis that progress of systemic autoimmune disease impairs parenchymal growth in the parietal cortex and hippocampus, likely by inducing neuronal loss.

In comparison with age-matched MRL +/+ control

[*] $t_{10} = 2.868$, $P = 0.017$, and

[**] $t_{13} = 2.238$, $P = 0.043$.

TABLE 3

SAB Rates (%) of 14-Week-Old MRL-lpr and Control MRL +/+ Mice Following 8 Weekly i.p. Injections With Immunosuppressive Drug Cyclophosphamide (100 mg/kg body weight) or Saline (SAL)[†]

Group	Acquisition	Reversal
MRL-lpr SAL (n = 12)	60 ± 3 [*]	50 (6/12)
MRL +/+ SAL (n = 10)	84 ± 7	90 (9/10)
MRL-lpr CY (n = 12)	75 ± 4	67 (8/12) [**]
MRL +/+ CY (n = 8)	83 ± 8	50 (4/8)

SAB, spontaneous alternation behavior; CY, cyclophosphamide; SAL, saline.

[†] Sustained immunosuppressive treatment reduced the substrain difference in SAB acquisition rate. However, this treatment appeared to have detrimental effects in the MRL +/+ CY group in the "reversal" form of the test.

[*] In comparison to MRL-lpr CY, $t_{22} = 2.691$, $P = 0.013$.

[**] Not statistically different from other groups in multiple comparisons using the Fisher test.