Immunosuppression prevents neuronal atrophy in lupus-prone mice: evidence for brain damage induced by autoimmune disease?

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Abstract

An early onset of systemic, lupus-like disease in MRL-lpr mice is accompanied by deterioration in their behavioral performance and atrophy of pyramidal neurons in the parietal cortex and the hippocampal CA1 area. Using the immunosuppressive drug cyclophosphamide (CY) to attenuate the disease, we have tested the hypothesis that the autoimmune/inflammatory process is responsible for changes in brain morphology. A modified Golgi impregnation method revealed that, in comparison to saline-treated controls, immunosuppressive treatment with CY (100 mg/kg/week i.p. over 8 weeks) increased dendritic branching and spine numerical density in the CA1 region of MRL-lpr mice and MRL+/+ mice, which develop less severe manifestations of the disease. More interestingly, CY selectively prevented the atrophy and aberrant morphology of pyramidal neurons in the parietal cortex of MRL-lpr mice. The neuropathological measures (in particular reduced dendritic spine density) significantly correlated with increased serum levels of antinuclear antibodies and splenomegaly. The present results support the hypothesis that chronic autoimmune disease induces functionally important changes in neuronal morphology, and provide an empirical basis for understanding the behavioral dysfunction in systemic lupus erythematosus and autoimmune phenomena reported in some forms of mental illness. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Autoimmunity; Inflammation; Dendrites; Neurotoxicity; Hippocampus; Parietal cortex; Golgi method; Lupus; MRL mice

1. Introduction

Similarly to human disease systemic lupus erythematosus [reviewed in (Denburg et al., 1993; Perry and Miller, 1992)], the spontaneous development of autoimmune manifestations in MRL/MpJ-lpr/lpr (MRL-lpr) mice is accompanied by a constellation of behavioral deficits, most consistently noted in emotional reactivity, affective behavior (Szechtman et al., 1997; Sakic et al., 1997), and spatial learning and memory (Hess et al., 1993; Sakic et al., 1992). The so-called ‘autoimmunity-induced behavioral syndrome’ (Sakic et al., 1997) coincides temporally with a progressive infiltration of lymphoid cells into the choroid plexus and brain parenchyma (Farrell et al., 1997), ventricular enlargement (Denenberg et al., 1992), and neurodegeneration. In particular, compared to congenic MRL/MpJ+/+ (MRL +/+) controls (which develop a less severe form of autoimmune disease), MRL-lpr mice show reduction in the branching of pyramidal neuron dendrites, atrophy of dendritic spines, and appearance of an aberrant pyramidal neuron morphology in the parietal cortex and hippocampus (Sakic et al., 1998a). Moreover, the course of neuronal atrophy in the MRL-lpr substrain is age-related, suggesting that the neuropathological process is linked either to accelerated aging or to the progression of the autoimmune disease (Theofilopoulos, 1992).

To examine a possible causal relationship between chronic immune activation and aberrant dendritic morphology, we used immunosuppressive treatment with cyclophosphamide. In our previous studies, prolonged treatment with CY effectively prevented infiltration of lymphoid cells into the MRL-lpr brain (Farrell et al., 1997) and reduced behavioral (Sakic et al., 1996; Sakic et al., 1995) and neurohormonal (Sakic et al., 1999) differences between the MRL substrains. Therefore, the overall expectation was
that generalized attenuation of immune hyperactivity would reduce, or even prevent, aberrant neuronal morphology. Indeed, the present results support the immune hypothesis by showing that appearance and complexity of dendrites on pyramidal neurons is normalized in immunosuppressed MRL-lpr mice.

2. Materials and methods

2.1. Animals

Three-week-old (±3 days) MRL/MpJ-lpr/lpr (MRL-lpr) and MRL/MpJ-+/+ (MRL+/+) male mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and habituated to the colony room and the experimenter (1 h/group/day) over 5 days during the fourth week of life. The mice were housed in groups of four per cage and under standard laboratory conditions (light phase: 8 A.M.–8 P.M., food and water ad lib). The experimental protocols were performed in accordance with rules and regulations of the Canadian Council of Animal Care.

2.2. Immunosuppressive treatment

The therapeutic effect of cyclophosphamide (CY) on the development of autoimmune/inflammatory manifestations has been previously demonstrated in our studies (Sakic et al., 1995; Sakic et al., 1996) and those of others (Grotta et al., 1990; Grotta et al., 1989; Shiraki et al., 1984). In addition to simply reducing leukocyte numbers (Snippe et al., 1976), CY also causes these cells to be unresponsive to stimuli, leading to immunosuppression (ten Berge et al., 1982). CY was injected weekly (100 mg/kg i.p.; mouse LD50, 405 mg/kg i.p.; ‘Procytox’, Homer, Montreal, Canada) to half of the mice in each group. The treatment started in the fifth week of life and ended on the 14th week. The other half of the animals received nine injections of an equivalent volume (approx. 0.2–0.3 ml) of saline (Sal). Mice were assigned into one of four groups, according to a strain (MRL-lpr vs. MRL+/+) and treatment (CY vs. Sal). To monitor for CY-induced attenuation in growth (Sakic et al., 1995), mice were matched for body weight at the beginning of the experiment and re-weighed during the sacrifice, using a digital scale (Sartorius PT 600, VWR Scientific of Canada Ltd.).

2.3. Indices of autoimmune disease

High levels of serum anti-nuclear antibodies (ANA) and splenomegaly are typical manifestations of systemic autoimmune, lupus-like disease (Theofilopoulos, 1992), and were examined here. Exsanguination and spleen extraction were performed 7 days after the last CY or saline injection. Using Natelson blood-collecting tubes (250 μl, Fisher-Scientific) for retro-orbital puncture, the blood samples (~1 ml) were collected under light (20–30 s) inhalation anaesthesia with isoflurane. Blood was left to coagulate in small plastic vials, and later centrifuged for 10 min at 3000 RPM. Serum was separated from the clot and stored at −20°C until further analysis. ANA concentration was measured using a sandwich ELISA Anti-Nuclear Antibodies kit, according to the manufacturer’s instructions (Alpha Diagnostic International Inc., San Antonio, TX). In brief, each serum sample (~3 μl) was diluted 1:100 with sample diluent (Cat. No. 5204) and incubated in an antigen coated and non-antigen coated well for 30 min at room temperature. Each well was aspirated, washed four times with 300 μl of diluted wash buffer (diluted 1:20 in distilled water) and 100 μl of diluted enzyme conjugate (goat anti-mouse IgG (H+L)-HRP; diluted 1:100 with sample diluent) was added into each well. The plate was covered, gently mixed and incubated for 30 min at room temperature. After that, wells were aspirated and washed five times with the wash buffer. To remove all the unbound enzyme conjugate, 100 μl of chromogenic substrate (TMB) was added into each well, mixed and incubated for 15 min at room temperature. The reaction was stopped with 100 μl of stop solution/well at the timed intervals. The absorbance was measured in an ELISA reader (at 450 nm) and specific antibody binding was assessed as the difference between antibody reactivity in antigen-coated and non-antigen-coated wells. The wet spleen weight was determined on an analytical scale (Sartorius 2024 MP, VWR Scientific of Canada Ltd.), immediately upon extraction.

2.4. Golgi-impregnation method

After intracardial perfusion with PBS, the brains were extracted and immediately immersed in 20 ml of Golgi-Cox solution (mixture of K2Cr2O7, HgCl2 and K2CrO7 water solutions). Twenty-four hours later they were dried and quickly weighed on an analytical scale. After 14 days in the Golgi-Cox solution, the brains were immersed in a 30% sucrose solution for 2–5 days. After fixation, the tissue was cut on a Vibratome (200 μm sections), and morphological analysis was performed by an experimenter blinded to the experimental design (Gibb and Kolb, 1998). Layer III pyramidal cells in Zilles’ area Par I were traced using a camera lucida at 250X. In order to be included in the data analysis, the dendritic trees of the pyramidal cells had to fulfill the following criteria: (a) the cell had to be well impregnated and not obscured by blood vessels, astrocytes, or heavy clusters of dendrites from other cells; (b) the apical and basilar arborizations had to appear to be largely intact and visible in the plane of section. The cells were drawn and analysed using two different procedures. In the first, each branch segment was counted and summarized by branch order (Coleman and Riesen, 1968). This method gives an estimate of the branching complexity (topography) of cells. Branch order was determined for the basilar dendrites such that branches originating at the cell
body were first order; those originating after one bifurcation, second order; and so on. Branch order was determined for the apical dendrites such that branches originating from the primary apical dendrite were first order and so on. A Sholl analysis of ring intersections was used to estimate dendritic length (Sholl, 1956). The number of intersections of dendrites with a series of concentric circles at 20 μm intervals from the center of the cell body was counted for each cell. Total dendritic length (in μm) can be estimated by multiplying the number of intersections by 20×. Cells were chosen by locating the parietal cortex (Zilles’ Par 1) at the level of the anterior commissure and then by drawing each cell in the section that met the criteria listed above. This region is distinct in Golgi-stained sections and normally it is possible to obtain sufficient cells from two adjacent sections. In the CA1 region of the hippocampus the basilar fields were measured for branching because they are clearly separated and meet the above criteria. Five cells were drawn in each hemisphere of each mouse, and statistical analysis was done by taking the mean. Spine density was measured from one apical dendritic branch in the terminal tuft, one secondary apical branch beginning about 50% of the distance between the cell body and terminal tuft, one basilar terminal branch, which was always a fourth order terminal branch, and one secondary basilar branch. Spine density measures were made from a segment greater than 10 μm in length, and usually about 50 μm. The dendrite was traced (1000×) using a camera lucida drawing tube and the exact length of the dendritic segment calculated by placing a thread along the drawing and then measuring the thread length. Spine density was expressed as the number of spines per 10 μm. No attempt was made to correct for spines hidden beneath or above the dendritic segment so the spine density values are likely to underestimate the actual density of the dendritic spines.

Golgi staining was initially performed on 15 brains, with 4 brains/substrain/treatment, except for the saline-treated MRL-lpr group (n=3), where one mouse died prematurely. Due to poor staining of the hippocampal areas and the apical branches in the cortical neurons in the first cohort, the experiment was repeated with a second cohort of 19 animals. Each group consisted of 5 brains/substrain/treatment, except the MRL +/+ group where n=4 (one mouse died during the CY administration). Therefore, the overall sample size was lower when hippocampal neurons and apical terminals were considered for statistical analysis. Wet brain weight was measured in the second cohort.

2.5. Statistics

Statistical analysis was performed using ANOVA with repeated measures, with substrain (MRL-lpr vs. MRL +/+), treatment (CY vs. Sal) and cohort (first vs. second) as between group factors, as well as hemisphere (left vs. right) or branch order as within subjects factors in the analysis of neuromorphological variables. Given 2×2 experimental design, Student’s t-test was used for post-hoc analysis. Pearson’s correlation was used to examine the relationship between autoimmune and neuromorphological measures. Significance level was set at P<0.05. All computations were performed using the SPSS 9.0 statistical package. Graphs show means±S.E.M.

3. Results

3.1. Immune measures

The immunosuppressive effect of CY was confirmed by lower serum ANA levels of CY-treated mice in both cohorts [treatment: F(1,26)=8.282, P=0.008; Fig. 1A]. CY treatment also reduced ANA levels within the MRL +/+ group [t(15)=3.107, P=0.007], its effect was more pronounced in the MRL-lpr group, as indicated by a profound decrease in ANA levels [substrain by treatment

Fig. 1. The effect of cyclophosphamide (CY) on indices of autoimmune disease. Prolonged treatment with CY (from 5 to 14 weeks of age) reduced serum levels of anti-nuclear antibodies (ANA) in both substrains (A), and prevented splenomegaly in MRL-lpr mice (B).
interaction: $F(1,26) = 4.356, P=0.047$] and reduced spleen weight in CY-treated MRL-lpr mice [substrain by treatment interaction: $F(1,26)=5.134, P=0.032$; Fig. 1B].

3.2. Body and brain weight

The MRL-lpr and MRL +/+ mice had comparable body weight at the time of sacrifice [MRL-lpr mice: 41.7±0.9 g vs. MRL +/+ mice: 39.9±0.9 g]. Although chronic CY-treatment was found to significantly reduce body weight in the previous studies (Sakic et al., 1995, 1996), the CY-treated mice did not significantly differ from Sal-treated mice [41.7±0.9 g in CY-treated mice vs. 39.9±0.9 g in Sal-treated mice], likely due to a smaller sample size used in the present study. The brain weights were comparable between the treatments [i.e., 637.4±4.85 mg in CY-treated mice vs. 649.4±7.34 mg in Sal-treated mice], and the previously observed (Sakic et al., 1998a) lower brain weight in autoimmune MRL-lpr mice has been confirmed [633.2±5.4 mg vs. 655.4±5.6 mg in the MRL +/+ group; treatment effect: $F(1,19) = 7.622, P=0.015$].

3.3. Neuromorphological measures

3.3.1. Hippocampus

As found previously (Sakic et al., 1998a), measures of dendritic morphology were reduced in MRL-lpr mice compared to the MRL +/+ controls [for Sholl crossings, substrain: $F(1,12)=24.525, P<0.001$; for basilar branching, substrain: $F(1,12)=69.592, P<0.001$; for basilar spine density, substrain: $F(1,12)=158.358, P<0.001$]. Importantly, CY treatment increased, in both substrains, dendritic length [for Sholl’s crossings branch orders 3–6, treatment: $F(1,12)=7.224, P=0.02$, Fig. 2A], total number of branches [treatment: $F(1,12)=12.43, P=0.004$, Fig. 2B], and spine density on the hippocampal pyramidal neurons [for basilar spines, treatment: $F(1,12)=21.95$, $P=0.001$, Fig. 2D]. The magnitude of the increase in apical spines was larger in MRL +/+ mice, as evidenced by significant substrain by treatment interaction [$F(1,12)=43.538, P<$.

![Fig. 2. The effect of CY on neuromorphological measures in the CA1 region of the hippocampus. The length, assessed as number of Sholl crossings (A) and complexity, assessed as number of dendritic basilar branches (B) was increased in both MRL substrains upon treatment with CY. Similarly, CY treatment increased the spine density in apical (C) and basilar regions (D).](image-url)
The number of Sholl crossings in apical and basilar dendrites did not differ across the substrains or treatments (data not presented). Also, the hemisphere and the cohort factor did not show a significant effect.

3.4. Correlation between immune and neuromorphological measures

The correlation between ANA levels and spleen weight was high (intercorrelation $r_{xy} = 0.856$, $P<0.001$). Table 1 shows the extent of correlation between each of these immune measures with measures of dendritic branching and spine density. As is evident, the pattern of correlations strongly suggested that the brain of mice with the most severe autoimmune manifestations showed the lowest density of spines and the smallest complexity in dendritic branching. A similar relationship was observed when only the MRL-lpr group of mice was analysed ($N=8$), with the most consistent correlations associated with spine density.

Fig. 3. The effect of CY on neuromorphological measures in the parietal cortex. The dendritic complexity in the apical (A) and basilar regions (B) was normalized in MRL-lpr mice upon treatment with CY, even exceeding the values in the MRL $+/+$ group in some cases. Similarly, CY treatment clearly normalized the spine density in both apical (C) and basilar regions (D) of the MRL-lpr neurons.
Fig. 4. Representative photomicrographs of apical terminals from pyramidal neurons (layer III) in the parietal cortex. (A) Sal-treated and (B) CY-treated MRL +/+ groups showing comparable spine density and morphology. (C) Sal-treated MRL-lpr group showing aberrant spine morphology at an advanced stage and (D) CY-treated MRL-lpr group showing normalized spine density and shape.

both in the hippocampus (e.g., with spleen weight $r = -0.967$ or with ANA $r = -0.847$) and parietal cortex (e.g., with spleen weight $r = -0.947$ or ANA $r = -0.905$).

4. Discussion

The present study reveals that early treatment with the immunosuppressive agent CY can prevent the expected neuronal atrophy of dendritic spines and branching in mice prone to systemic autoimmune disease. Moreover, it shows that the extent of dendritic atrophy, and especially dendritic spine loss, is highly correlated with the severity of autoimmune manifestations. Together with our previous findings that neuromorphological changes and the progress of autoimmune disease follow a similar time course (Sakic et al., 1998a), these results support the hypothesis that it is the onset of lupus-like disease which induces a neurodegenerative process in the pyramidal neurons. As such, neuronal degeneration may represent one of the principal factors accounting for the neurobehavioral dysfunction seen in autoimmune animals (Sakic et al., 1997).

Table 1

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<thead>
<tr>
<th></th>
<th>ANA level</th>
<th>Spleen size</th>
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<tr>
<td>Hippocampus (N=16)</td>
<td></td>
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<tr>
<td>Basilar crossings</td>
<td>$-0.776^{***}$</td>
<td>$-0.671^{**}$</td>
</tr>
<tr>
<td>Basilar branching</td>
<td>$-0.897^{***}$</td>
<td>$-0.797^{***}$</td>
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<tr>
<td>Apical spine density</td>
<td>$-0.639^{**}$</td>
<td>$-0.568^{*}$</td>
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<tr>
<td>Basilar spine density</td>
<td>$-0.956^{***}$</td>
<td>$-0.869^{***}$</td>
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<tr>
<td>Parietal cortex (N=31)</td>
<td></td>
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<tr>
<td>Basilar branching</td>
<td>$-0.441^{*}$</td>
<td>n.s.</td>
</tr>
<tr>
<td>Apical spine density</td>
<td>$-0.722^{**}$</td>
<td>$-0.882^{***}$</td>
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<tr>
<td>Basilar spine density</td>
<td>$-0.685^{***}$</td>
<td>$-0.626^{***}$</td>
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| $*$, $P<0.05; **$, $P<0.01; $$$$, $P<0.001; $n.s.-not significant.|

4.1. Neuroprotective effect of CY

The present results are consistent with the reported neuroprotective effects of CY in autoimmune conditions (Tajti et al., 1991; Roth et al., 1982) and viral infections (Lipton and Canto, 1977; Townsend and Baringer, 1979). However, the mechanism underlying the enhancing effect of immunosuppression on dendritic complexity in the CA1 region requires clarification. Hippocampal neurons, and those cultured from the CA1 area in particular (Mattson and Kater, 1989), are highly sensitive to metabolic insults (Sapolsky, 1996; Sapolsky, 1996; McEwen, 1999) and immune activation (Reul et al., 1994). Therefore, it is possible that the early serologic manifestations [e.g., upregulation in production of cytokines (Tang et al., 1991)] had affected these vulnerable neurons (Campbell et al., 1993; Qiu et al., 1998) both in diseased MRL-lpr and the less symptomatic MRL +/+ mice. This possibility may be supported by the evidence that in the CY-treated MRL +/+ controls ANA levels were the lowest, suggesting minimal autoimmune activity and thus reduced exposure to potentially harmful immunoenocrine factors. An intriguing question is how CY, an anti-proliferative and teratogenic drug (Singh et al., 1974), prevents atrophy of
neuronal dendrites in autoimmune animals. Although this cannot be resolved by the present set of data, we discuss two possibilities below.

4.2. Immune pathway

CY is a cytotoxic alkylating agent, used in cancer chemotherapy and as an immunosuppressive agent. Activation occurs in the liver, and its active metabolites bind to DNA, preventing the ‘unzipping’ required for proliferation (Snippe et al., 1976). A direct effect of CY on brain tissue seems unlikely, since CY enters the CNS (Neuwelt et al., 1984) in a subtherapeutic concentrations (Genka et al., 1990), and does not change neuronal morphology in vitro (Nicoli et al., 1998). An alternative explanation would be that CY reduces infiltration of cytotoxic lymphocytes into the brain and/or normalizes activity of the hypothalamus-pituitary-adrenal (HPA) axis. Regarding the immune mechanism, it is known that daily injection of 100 mg/kg of CY reduces the number of circulating T and B cells, lowers serum levels of immunoglobulins and depresses primary and secondary antibody responses in patients with multiple sclerosis (ten Berge et al., 1982). There is ample evidence that circulating lymphocytes and mononuclear cells penetrate through the damaged bloodbrain barrier (Vogelweid et al., 1991) and infiltrate the choroid plexus and brain parenchyma of young MRL-lpr mice (Farrell et al., 1997; Hess et al., 1993; Alexander et al., 1983). Therefore, it is likely that chronic CY treatment reduced the number and activity of circulating leukocytes which (upon entering the brain) may synthesize brain-reactive antibodies (Crimando and Hoffman, 1992; Hoffman et al., 1987; Sakic et al., 1993), cytokines (Campbell et al., 1993) and other metabolites (Piani et al., 1992; Merrill and Murphy, 1997) potentially harmful to neuronal development, maturation, and survival (Rothwell and Relton, 1993; Wollman et al., 1992). The evidence that CY was specific in reducing infiltration of CD45-positive cells (Farrell et al., 1997) and that prolonged treatment with anti-CD4 antibody prevented the accumulation of CD4-positive cells in the brain tissue of MRL-lpr mice (O’Sullivan et al., 1995) supports the hypothesis that neuromorphological damage is induced by infiltration of circulating immune cells.

4.3. Endocrine pathway

With respect to the endocrine pathway, it has been shown that chronic elevation in corticosterone production induces neuronal atrophy in hippocampal regions (Sapolsky, 1996; Sapolsky et al., 1988; Woolley et al., 1990). If so, corticosterone may be one of the neuropathogenic factors in MRL-lpr mice, given that this strain is prone to a sustained spontaneous increase in basal corticosterone levels (Lechner et al., 1996; Hu et al., 1993). It is unlikely that the CY treatment per se had an effect in the present study because there is no evidence that injections of CY affect corticosterone production (Roudebush and Bryant, 1991; Fast et al., 1982). Rather, one may assume that generalized immunosuppression reduced exposure of the HPA axis to neuroactive and pro-inflammatory cytokines (Turnbull and Rivier, 1999). This may have had a further impact both on adrenal activity, and on normalization of glucocorticoid production, which are considered to be parts of one of the primary modulatory systems of immune function (McEwen et al., 1997). However, more specific interventions are required to elucidate the pathogenic mechanisms that directly produce neuronal atrophy.

4.4. Autoimmunity-induced neurodegeneration: relevance to neuropsychiatric disorders

Given the progressive development of the autoimmunity-induced behavioral syndrome in MRL-lpr mice (Sakic et al., 1997; Szechman et al., 1997), reduced dendritic complexity may be one of the initial markers of the structural damage subsequently associated with excessive cell death (Sakic et al., 2000) and ventricular enlargement (Denenberg et al., 1992). Together with imbalances in hormonal production (Sakic et al., 1999; Sakic et al., 1998b) these neuromorphological changes may be the basis of the profound deficits in emotional reactivity (Sakic et al., 1994) and cognitive performance of autoimmune mice (Hess et al., 1993). Neuropathological and behavioral changes in autoimmune MRL-lpr mice are a unique natural preparation in which autoimmunity affects brain morphology and behavior. The MRL model is being developed to better understand neuropsychiatric lupus erythematosus, and thus far has shown high face and construct validity (Denburg et al., 1999; Szechman et al., 1997; Sakic et al., 1997). The present results also support the causal relationship between autoimmune phenomena (McGeer et al., 1994; Forster and Lal, 1991; Singh et al., 1997; Zimmerman et al., 1993; Singh et al., 1993; Ganguli et al., 1994) and reduced spine density in mental disorders such as Alzheimer’s disease (el Hachimi and Foncin, 1990; Einstein et al., 1994), autism (Raymond et al., 1996) and schizophrenia (Garey et al., 1998). Therefore, elucidation of autoimmunity-associated neurodegeneration and behavioral dysfunction in MRL-lpr mice may become novel preparation in understanding the role of chronic immune activation in some forms of human mental illness (Muller and Ackenheil, 1998).

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