Recombinant T Cell Receptor Ligand Treats Experimental Stroke

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Background and Purpose—Experimental stroke induces a biphasic effect on the immune response that involves early activation of peripheral leukocytes followed by severe immunodepression and atrophy of the spleen and thymus. In tandem, the developing infarct is exacerbated by influx of numerous inflammatory cell types, including T and B lymphocytes. These features of stroke prompted our use of recombinant T cell receptor ligands (RTL), partial major histocompatibility complex Class II molecules covalently bound to myelin peptides. We tested the hypothesis that RTL would improve ischemic outcome in the brain without exacerbating defects in the peripheral immune system function.

Methods—Four daily doses of RTL were administered subcutaneously to C57BL/6 mice after middle cerebral artery occlusion, and lesion size and cellular composition were assessed in the brain and cell numbers were assessed in the spleen and thymus.

Results—Treatment with RTL551 (I-A\(b\) molecule linked to MOG-35-55 peptide) reduced cortical and total stroke lesion size by approximately 50%, inhibited the accumulation of inflammatory cells, particularly macrophages/activated microglial cells and dendritic cells, and mitigated splenic atrophy. Treatment with RTL1000 (HLA-DR2 moiety linked to human MOG-35-55 peptide) similarly reduced the stroke lesion size in HLA-DR2 transgenic mice. In contrast, control RTL with a nonneuroantigen peptide or a mismatched major histocompatibility complex Class II moiety had no effect on stroke lesion size.

Conclusions—These data are the first to demonstrate successful treatment of experimental stroke using a neuroantigen-specific immunomodulatory agent administered after ischemia, suggesting therapeutic potential in human stroke. (Stroke. 2009;40:2539-2545.)

Key Words: autoreactive T cells ■ immunotherapy ■ recombinant TCR ligands ■ stroke

The role of inflammatory factors and their contribution to tissue injury in stroke has been well established. After dense cerebral ischemia, there is rapid accumulation of inflammatory cells associated with breakdown of the blood–brain barrier.1-3 These infiltrating cells, including neutrophils, macrophages, and lymphocytes,4 exacerbate the evolving ischemic injury. Although the importance of lymphocytes and adaptive immunity is not well-defined, mice lacking T and B cells sustain smaller infarcts than normal mice.5,6 Furthermore, cerebral ischemia engages the systemic immune system, first by massive activation, then by immunosuppression marked by spleen and thymus atrophy and loss of immunocompetent cells.7-11 Accordingly, successful immunotherapy after ischemia would need to reduce inflammatory damage and concomitantly support survival of peripheral immunity.

Recombinant T cell receptor (TCR) ligands (RTL) are partial major histocompatibility complex (MHC) Class II molecules comprised of covalently linked \(\beta1\) and \(\alpha1\) chains tethered to antigenic peptides. These unique molecules were designed as minimal ligands for the TCR of peptide-specific T cells. Unlike 4-domain MHC II molecules that can induce T cell activation and apoptosis,12 RTLs are partial agonists that deviate autoreactive T cells to become nonpathogenic.13,14 We previously demonstrated that RTL could prevent and/or reverse clinical signs of experimental autoimmune encephalomyelitis,15-17 collagen-induced arthritis,18 and experimental autoimmune uveitis.19 Recent studies with RTL551 (I-A\(b\) molecule covalently tethered to mouse myelin oligodendrocyte glycoprotein mMOG-35-55 peptide) showed dramatic reversal of MOG-35-55-induced experimental auto-

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immune encephalomyelitis, reduction of pathogenic central nervous system (CNS) cells, downregulation of endothelial cell adhesion molecules (intercellular adhesion molecule and vascular cell adhesion molecule) and broad reduction of CNS chemokines/receptors.20

One prevailing hypothesis is that cerebral ischemia breaches the blood–brain barrier, thereby exposing CNS antigens and facilitating development of anti-CNS responses.21 Recent reports suggest that induction of T cell tolerance to CNS antigens by mucosal administration of myelin basic protein or mMOG-35-55 peptide can reduce infarct size and CNS autoimmunity and improve clinical outcome in mice with experimental stroke.22–25 Based on these observations and RTL effects on experimental autoimmune encephalomyelitis, we hypothesized that treatment of experimental stroke with RTL551 would reduce infarction volume through a neuroantigen-specific mechanism.

Materials and Methods

Animals

The study was conducted in accordance with National Institute of Health guidelines for the use of experimental animals, and the protocols were approved by the Institutional Care and Use Committee. All experiments used age-matched, sexually mature (20 to 25 g) male mice (C57BL/6, Charles River, Wilmington, Mass; or HLA-DRB1*1502 mice obtained from Dr Chella David, described previously).26-27 The mice were bred and housed at the Portland Oregon Veterans Affairs Medical Center according to institutional guidelines.

RTL Construction and Production

RTL molecules consist of the α1 and β1 domains of MHC II molecules expressed as a single polyepitope with or without antigenic amino terminal extensions28-29: RTL551 = I-Aβ linked to mouse MOG-35-55 peptide (MEVGWYRSPFSRVVHLYRNGK); RTL553 = recombinant I-Aβ linked to 1-Ea-52-68 (3Kp) peptide (ASPEAKAQKANKAVD30,31); RTL342M = HLA-DR2 linked to mouse MOG-35-55 (mMOG35-55) peptide; and RTL1000 = HLA-DR2 linked to human MOG-35-55 (hMOG35-55) peptide (MEVGYWRPPFSRVVHLYRNGK). RTL were constructed de novo or by sequential site-directed mutagenesis of previous constructs.20 Protein purification was as previously described28,29 with a 30- to 40-mg yield of purified protein per liter of bacterial cell culture.

Treatment With RTL

Mice were randomized to treatment with RTL551 0.1 mL (1 mg/mL, subcutaneous injection at onset of reperfusion followed by doses at 24, 48, and 72 hours of reperfusion for a total of 4 treatments), RTL553, RTL342M, RTL1000, or vehicle (Tris-HCl, pH = 8.5). To determine if RTL551 altered arterial blood pressure or blood gases, separate cohorts of mice were dosed with RTL551 (n = 3) or vehicle (n = 3) and then continuously monitored for 2 hours. Arterial blood gases, osmolarity, and electrolytes were also measured in these cohorts at 2 hours posttreatment.

Transient Middle Cerebral Artery Occlusion Model

Transient middle cerebral artery occlusion (MCAO) was performed in isoflurane-anesthetized male mice using 60 minutes of intraluminal filament occlusion as described.6-7 Temporalis muscle temperature was maintained at 35.5°C to 37.5°C throughout MCAO surgery with warming lights. Adequacy of occlusion was confirmed by intraschismic laser-Doppler flowmetry (Moor Instruments Ltd, Oxford, UK). Sham-operated mice experienced identical surgical procedures except for vascular opening and filament insertion. Tissue was harvested under deep anesthesia at 96 hours of reperfusion.

Cerebral infarct size was determined by standard triphenyltetrazolium chloride (Sigma Aldrich) staining and digitally quantified by Sigma Scan Pro5 software (Jandel, San Rafael, Calif) as described.7 In additional experiments, mice were saline-perfused through the ascending aorta to remove nonparenchymal cells. The brain was divided into the ischemic right hemisphere and nonischemic left hemisphere and then dissociated mechanically through a 150-mesh screen. CNS mononuclear cells were isolated by Percoll gradient centrifugation as described.32

Isolation of Cells From the Spleen, Thymus, and Blood

The spleen and thymus were isolated from vehicle- and RTL-treated sham and stroke mice. Single cell suspensions were prepared by passing the tissue through a 100-μm nylon mesh screen. Cells were washed using RPMI and red cells were lysed using 1× red cell lysis buffer (eBioscience, San Diego, Calif). The cells were washed twice, counted, and resuspended in stimulation medium containing 10% fetal bovine serum. For real-time polymerase chain reaction, splenocytes were pelleted, snap-frozen, and stored at −80°C until tested. For fluorescence activated cell sorting staining, cells were washed with staining medium (1× phosphate-buffered saline, 0.5% bovine serum albumin, 0.02% sodium azide) before adding specific antibodies. Cardiac blood was collected in 3 mg/mL EDTA, and the cells were pelleted after lysis of red cells, washed, counted, and resuspended in staining medium for fluorescence activated cell sorting staining.

Analysis of Cell Populations by Fluorescence Activated Cell Sorting

Four-color (fluorescein isothiocyanate conjugated, phycoerythrin (PE), propidium iodide (PI), allophycocyanin) fluorescence flow cytometric analyses were performed to determine the phenotypes of brain and blood mononuclear cells. Cells were washed with staining medium and stained with a combination of the following mAbs: CD3e (145-2C11), CD4 (L3T4), CD8 (Ly-2), CD11b (M1/70), CD11c (HL-3), and CD19 (1D3) for 20 minutes on ice. After incubation with mAbs, cells were acquired with a FACS Calibur (BD Biosciences). Forward and side scatter parameters were chosen to identify lymphocytes. Dead cells were gated out using propidium iodide discrimination. Data were analyzed using FCS express software (De Novo). For each experiment, cells were stained with appropriate isotype control Abs to establish background staining and to set quadrants before calculating the percentage of positive cells.

Statistical Analysis

Statistical differences between parameters assessed in vehicle and RTL groups were determined by Student t test. Values of P < 0.05 were considered significant. Data are presented as means±SD.

Results

RTL551 Treatment Significantly Reduces Infarction Size After 96 Hours Reperfusion

Treatment with RTL551 did not alter arterial blood pressure over a 2-hour observation window, and there were no differences at 2 hours as compared with vehicle-treated mice (Veh; RTL 80±6 mm Hg; Veh 75±8, n = 3 per group). Similarly, there were no differences in arterial blood pH (RTL 7.33±0.03; Veh 7.34±0.02), pCO2 (RTL 49±5 mm Hg; Veh 41±5) or pO2 (RTL 160±17 mm Hg; Veh 178±4). Plasma electrolytes, glucose, and osmolarity were also different in RTL versus vehicle treated-groups (data not shown). Four daily treatments with RTL551 treatment resulted in approximately 50% reduction in infarction volume in the cortex (49.1±12.6, Veh versus 25.6±18.4, RTL-treated mice, P = 0.009) but not in the striatum (Figure 1A). Total infarction in RTL551-treated mice was
26.5±12.4 versus 39.7±11.0 in vehicle-treated controls (P=0.04). These data demonstrate that RTL551 can successfully reduce the cortical and total stroke lesion volumes when administered after induction of MCAO.

RTL Treatment of Stroke Requires a Neuroantigen Peptide and Matched MHC Moiety
To evaluate peptide specificity, C57BL/6 mice were treated with RTL553, which has the same MHC moiety as RTL551 (I-Ab) but is linked to a nonneuroantigenic peptide (I-Ea-52-68). As shown in Figure 1B, 4 daily treatments of MCAO mice with RTL553 had no effect on infarction volume in the cortex or striatum (39.8±23.4 in cortex and 93.1±9.0 in striatum). To address the contribution of RTL MHC II domains to therapy, MCAO mice were treated with RTL342M, which has the same mMOG-35-55 peptide as RTL551 but a different MHC II moiety (HLA-DR2 versus I-Ab). Similar to RTL553, treatment of MCAO with RTL342M failed to reduce infarction volume (Figure 1C). These data demonstrate that successful RTL treatment of stroke requires a neuroantigenic peptide (mMOG-35-55) and the autologous MHC moiety for the treated mice.

RTL1000 Reduces Infarction Size in DR2-Tg Mice
We further evaluated the therapeutic activity of humanized RTL1000, containing the HLA-DR2 moiety covalently linked to hMOG-35-55 peptide, on MCAO in DR2 mice. As shown in Figure 1D, 4 treatments of RTL1000 significantly reduced cortical infarction volume (27.7±18.2, Veh versus 5.3±5.2, RTL1000-treated mice). These data demonstrate that RTL1000, containing a neuroantigenic peptide and a matched MHC II moiety, can significantly reduce stroke lesion size in the cortex of a different mouse strain, further documenting the RTL treatment parameters established here.

RTL551 Inhibits Influx of Inflammatory Cells, Particularly Activated Microglia and Macrophages, in Ischemic Brain
We hypothesized that RTL551 treatment would reduce inflammatory cell infiltration into the brain after stroke. In fact, at 96 hours post-MCAO, RTL551 treatment reduced recovery of total brain mononuclear cells by 64% (from 220 000 to 80 000/mouse) and viable leukocytes by 52% (from 39 600 to 19 200/mouse) from the ischemic ipsilateral hemisphere with much lower cell counts (approximately 60 000 total brain cells and approximately 10 000 viable leukocytes) recovered from the unaffected contralateral hemisphere from both vehicle- and RTL-treated mice. Using 4-color flow cytometry, we found comparable percentages of most cell types in ischemic and unaffected hemispheres from vehicle- and RTL-treated mice. However, RTL551 treatment markedly downregulated CD45hiCD11bhi cells that represent macrophages and activated microglia in the ischemic right hemisphere of the brain (44% in vehicle-treated mice versus 15% in RTL551-treated mice; Figure 2A). Considering absolute numbers of recovered viable leukocytes, the reduction of CD45hiCD11bhi cells after RTL551 treatment (83%) was even more dramatic (from 17 424 to 2880/mouse; Figure 2B; Table 1). RTL551 treatment also caused a similar reduction (81%) in dendritic cells (DCs) and less pronounced reductions in absolute numbers of the other cell populations in the ischemic brain (25% to 59%; Table 1). In contrast, there were much lower baseline levels of these populations in the left nonischemic side of the brain (1.4% to 26%) that were relatively less affected by RTL551 treatment (23% to +137%; Table 1).
RTL Treatment Partially Restores Splenic But Not Thymic Cell Numbers After MCAO

To evaluate the effects of RTL551 treatment on stroke-induced atrophy, cell numbers in the spleen and thymus were counted in postischemic vehicle- and RTL-treated mice and compared with their respective sham-operated controls. As expected, MCAO induced a significant decrease in spleen and thymus cell numbers in the vehicle-treated group (Figure 3). Interestingly, viable cell counts were significantly increased in spleens of RTL551-treated versus vehicle-treated mice (4.6 ± 3.5, Veh- versus 17.1 ± 17.3, RTL-treated mice, P = 0.02) 96 hours after reperfusion. In contrast, treatment with RTL553 or RTL342M did not improve spleen cell counts (data not shown). The drastic reduction in thymic cell counts induced by MCAO was not altered by either RTL551 (Figure 3) or RTL553 (not shown) versus vehicle treatment.

RTL551 Treatment Does Not Affect the Distribution of Cell Subtypes in the Periphery

Given the partial restoration of spleen cell numbers in RTL551-treated mice, we tracked survival of specific splenic cell types. As shown in Table 2, RTL treatment did not affect the percentages of T cells, B cells, macrophages, or DCs in the spleen, although DCs were decreased in vehicle-treated MCAO versus sham-operated mice. As shown in Table 3, we confirmed our previous finding that MCAO increased circulating CD11b+ macrophages in vehicle-treated MCAO versus sham-operated mice and further noted that RTL treatment

Table 1. Example of Absolute Numbers Infiltrating Cells per Brain in the Left (Nonischemic) and Right (Ischemic) Hemispheres of Vehicle (n = 1 set of 2 Brains) and RTL551 (n = 1 set of 3 Brains) Treated Mice 96 Hours After MCAO

<table>
<thead>
<tr>
<th></th>
<th>Left (Nonischemic)</th>
<th>Right (Ischemic)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle RTL551</td>
<td>Percent Reduction</td>
</tr>
<tr>
<td>CD3 (T cells)</td>
<td>97</td>
<td>230</td>
</tr>
<tr>
<td>CD11b (resident microglia/macrophages)</td>
<td>7895</td>
<td>6874</td>
</tr>
<tr>
<td>CD45hiCD11b+ (activated microglia/macrophages)</td>
<td>248</td>
<td>259</td>
</tr>
<tr>
<td>CD11c (DC)</td>
<td>75</td>
<td>77</td>
</tr>
<tr>
<td>B220 (B cells)</td>
<td>2106</td>
<td>1402</td>
</tr>
<tr>
<td>DX5 (NK cells)</td>
<td>22</td>
<td>38</td>
</tr>
</tbody>
</table>
Table 2. Distribution of Cell Phenotypes in the Spleen of Vehicle and RTL551-Treated Mice 96 Hours After MCAO

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Vehicle</th>
<th>RTL551-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>MCAO</td>
<td>Sham</td>
</tr>
<tr>
<td>n=6</td>
<td>n=12</td>
<td>n=7</td>
</tr>
<tr>
<td>CD3+</td>
<td>35.1±8.8</td>
<td>48.8±12.7</td>
</tr>
<tr>
<td>CD4+</td>
<td>21.6±4.8</td>
<td>26.9±7.5</td>
</tr>
<tr>
<td>CD8+</td>
<td>13.5±3.6</td>
<td>20.1±5.8</td>
</tr>
<tr>
<td>CD11b+</td>
<td>8.5±5.8</td>
<td>5.2±1.9</td>
</tr>
<tr>
<td>CD11c+</td>
<td>2.4±1.2</td>
<td>0.6±0.5*</td>
</tr>
<tr>
<td>CD19+</td>
<td>46.9±2</td>
<td>36.7±11.8</td>
</tr>
<tr>
<td>Pl+</td>
<td>13.4±1.6</td>
<td>20.1±12.1</td>
</tr>
</tbody>
</table>

*Significant difference compared with RTL or vehicle-treated, sham-operated mice.

**Table 3. Distribution of Cell Phenotypes in the Blood of Vehicle and RTL551-Treated Mice 96 Hours After MCAO**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Vehicle</th>
<th>RTL551-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>MCAO</td>
<td>Sham</td>
</tr>
<tr>
<td>n=3</td>
<td>n=9</td>
<td>n=4</td>
</tr>
<tr>
<td>CD3+</td>
<td>16.8±7.4</td>
<td>11.6±3.0*</td>
</tr>
<tr>
<td>CD4+</td>
<td>9.3±4.2</td>
<td>5.4±1.5*</td>
</tr>
<tr>
<td>CD8+</td>
<td>7.6±2.5</td>
<td>6.2±1.8</td>
</tr>
<tr>
<td>CD11b+</td>
<td>18.8±6.9</td>
<td>39.2±14.1*</td>
</tr>
<tr>
<td>CD11c+</td>
<td>1.0±0.3</td>
<td>1.0±1.7</td>
</tr>
<tr>
<td>CD19+</td>
<td>43.0±9.1</td>
<td>23.4±12.7*</td>
</tr>
</tbody>
</table>

*Significant difference compared with RTL or vehicle-treated, sham-operated mice.

our study demonstrates for the first time that treatment with RTL after onset of MCAO reduces cortical and total infarct size, inhibits infiltrating inflammatory cells, particularly activated macrophages/microglial cells and DCs, into postischemic brain, and partially preserves spleen cell numbers that are typically ablated after MCAO. This result was specific to RTL551 treatment in C57BL/6 male mice and verified using a “humanized” RTL1000 construct to treat MCAO in HLA-DR2-Tg mice. The results clearly show that the therapeutic activity of RTL requires a neuroantigen peptide (mouse or human MOG-35-55) tethered to an MHC moiety that closely matches the Class II of the treated mouse strain (I-A^b for C57BL/6 mice and HLA-DR2 for DR2-Tg mice). In contrast, treatment of C57BL/6 mice with RTL553 comprised of I-A^b coupled to I-Ea-52-68 (a nonneuroantigen peptide) and RTL342M comprised of HLA-DR2 (nonmatched MHC Class II) coupled to mMOG-35-55 peptide did not have therapeutic effects.

Beyond the RTL-bound MOG-35-55 peptides that are known to induce inflammation in C57BL/6 and DR2 mouse strains,20,27 it is unknown what other neuroantigen peptides might also be effective in treating MCAO. It seems likely that the key features for therapeutic activity in stroke are antigenicity in combination with self MHC II molecules and expression in infarcted areas of the brain. This important issue awaits additional study. The need for a matched MHC moiety in the treating RTL suggests that therapeutic activity may require direct ligation of neuroantigen-reactive T cells by the intact RTL molecule rather than internalization and representation of the RTL-bound MOG peptide by host APC.

The influx of inflammatory cells into the brain after infarct was evaluated in detail in a previous study.4 Although T cells, B cells, neutrophils, and macrophages were all present, the predominant infiltrating population observed at 48, 72, and 96 hours after occlusion was the macrophage/activated microglial cell population that can be discerned by fluorescence activated cell sorting as CD45^hiCD11^hi. This population in the right brain hemisphere, but particularly inhibited (by $>80\%$) the infiltration of CD45^hiCD11b^hi macrophages/activated microglial cells and CD11c DCs. This result in stroke is quite similar to the previously-described effect of RTLS in blocking cellular infiltration into the CNS during experimental multiple sclerosis.20 This decrease in emigrating cells from the periphery may also help to explain RTL-induced preservation of spleen cell numbers during the second immunosuppressive stage of stroke.

The major unresolved question regarding the ability of RTL to inhibit lesion size after MCAO is establishing the conceptual framework for how it might work. The developing lesion in experimental stroke is propagated in part by infl-
tration of inflammatory cells, including T cells, B cells, neutrophils, and activated macrophages. Our previous studies demonstrated reduced cortical stroke lesion size in T and B cell-deficient SCID mice, and studies by others implicated T cells but not B cells or neutrophils as necessary inflammatory contributors. Moreover, mucosal induction of T cell tolerance to myelin antigens or transfer of tolerized T cells to naïve mice undergoing MCAO resulted in reduced infarct size and number of interferon-γ-producing cells and an increase in cells secreting interleukin-10 and transforming growth factor-β.21–23

Our working hypothesis is that normal brain antigens such as MOG are released in higher quantities in the stroke lesion and presented to the immune-infiltrating cells in an inflammatory environment, probably after ingestion of damaged brain tissue by phagocytic cells such as microglia, macrophages, and DCs. The rapid brain-to-spleen signaling that increases general activation of peripheral immune cells within 6 hours of reperfusion might allow activated T cells of many different specificities to infiltrate the stroke lesion site. Those T cells that are specific for MOG or other brain antigens might then be triggered to recruit other inflammatory cells into the lesion and thereby increase damage to the surrounding tissues. This scenario represents, to some degree, a native ability of T cells to recognize and respond to self-antigens, not dissimilar in concept to the natural IgM antibodies specific for nonmuscle myosin heavy chain Types II A and C implicated in reperfusion injury in skeletal and intestinal reperfusion injury described by Zhang et al. It seems likely that RTL treatment induces T cell tolerance to MOG-35-55 peptide in vivo through a different mechanism but with the same outcome as that induced by mucosal administration of myelin basic protein22 or MOG-35-55 peptide.23 We established previously that RTL specifically targets myelin-specific T cells and profoundly changes their functional properties from proinflammatory to anti-inflammatory cells that secrete interleukin-10, interleukin-13, and transforming growth factor-β.16 As such, the RTL-“tolerized” MOG-specific T cells could inhibit entry of other T cells into the CNS, prevent release of proinflammatory cytokines at the stroke lesion site, and reduce infiltration of activated macrophages/macrogial cells. This explanation for RTL inhibition of stroke is supported by our previous observation that mice pretreated with RTL before immunization with neuroantigens are profoundly protected against subsequent induction of experimental autoimmune encephalomyelitis. Moreover, by short-circuiting early brain-to-spleen activation, RTL modulation of MOG-specific T cells may have contributed to the observed partial preservation of spleen cell numbers. In summary, we demonstrated therapeutic activity of myelin antigen-specific immunomodulatory agents (recombinant TCR ligands, RTL551 and RTL1000) that were effective when administered after induction of MCAO. RTL therapy greatly reduced influx of inflammatory cells, particularly macrophages and DCs, into the ischemic hemisphere and partially prevented splenic atrophy that accompanies the downstream immunosuppressive phase of stroke. Future studies are underway to determine how long after MCAO the RTL can be given and still exert therapeutic benefit. RTL1000, a humanized RTL comprised of MOG-35-55 peptide covalently linked to an HLA-DR2 moiety, is currently in clinical trials in multiple sclerosis and potentially might be useful for treatment of human patients with stroke.

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