Lymphocytes
Potential Mediators of Postischemic Injury and Neuroprotection

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Abstract—Antigen-nonspecific inflammation appears to contribute to postischemic brain injury. Because there is a breach in the integrity of the blood-brain barrier after stroke, the immune system encounters novel central nervous system (CNS) antigens that allow for the development of a CNS antigen-specific autoimmune response. The nature of the immune response generated on antigen encounter is determined by the microenvironment at the site of antigen encounter. For instance, a systemic inflammatory response, such as that which would accompany an infection, could alter the microenvironment in such a way as to promote the initiation of deleterious autoimmunity. If patients who develop an infection in the immediate poststroke period are predisposed toward a CNS autoimmune response, it might help to explain why infection after stroke is associated with increased disability. We present data to support this hypothesis and to show that the breach in the blood-brain barrier can also be capitalized on to modulate the immune response to create a neuroprotective environment after stroke. (Stroke. 2007;38[part 2]:783-788.)

Key Words: immune response ■ inflammation ■ stroke ■ tolerance ■ lipopolysaccharide ■ myelin basic protein

A n inflammatory response occurs within the brain after stroke, and modulation of this inflammatory response improves outcome in experimental models of cerebral ischemic injury.1 Clinical trials of immune system modulation therapy after stroke, however, have not yet proved successful.2–4 The lack of clinical success does not necessarily mean that the immune response does not contribute to postischemic brain injury, but it does imply that our approach to controlling this immune system response may be flawed.5 Traditionally, “modulation” of the immune system response is accomplished by administration of immunosuppressive drugs that interfere with the development of an immune response. An alternative and perhaps more thoughtful approach to modulating the immune response is to “reeducate” the immune system to respond in a fundamentally different manner.

The inflammatory response that occurs after stroke is antigen nonspecific and mediated by the innate immune system. The innate immune system responds to bacterial pathogens through pattern recognition receptors (PRRs) known as toll-like receptors (TLRs) and nucleotide-binding oligomerization domains.6,7 PRRs recognize highly conserved structural motifs expressed by pathogens, but endogenous substances, such as heat shock proteins, also are capable of activating PRRs and may account for the inflammatory response that occurs after tissue injury.8–10 Whereas innate immunity is important in the defense against invading pathogens, activation of the innate immune response after ischemic brain injury could be detrimental. That there is an antigen-nonspecific inflammatory response mediated by lymphocytes is supported by a number of studies.11–13 Whether or not an adaptive immune response occurs is less clear.

The adaptive immune response is an antigen-specific response that requires the immune system be “educated to” and “remember” a given antigen. The results of this education depend on the characteristics of the microenvironment in which the antigen was encountered. For a T lymphocyte to become activated to a given antigen, the cell must “see” that antigen in the context of the major histocompatibility complex and receive an additional costimulatory signal. In general, this interaction leads to a Th1 immune response on future encounters with the antigen. A Th1 immune response is characterized by the secretion of proinflammatory cytokines (interleukin [IL]-2, IL-12, tumor necrosis factor-α, interferon [IFN]–γ) that promote the cellular immune response. Under normal circumstances, central nervous system (CNS) antigens are compartmentalized from the systemic immune system by the blood-brain barrier (BBB), so the immune system does not interact with and does not respond to these antigens. If the lymphocyte sees its cognate antigen but does not receive an appropriate costimulatory signal, it may remain “ignorant” of that antigen or become “tolerized” to it. Additionally, signals that serve to inhibit lymphocyte activation can be delivered to the cell, resulting in antigen-specific tolerance; on future encounters with that antigen, a Th2/Th3-type immune response may occur. A Th2/Th3 immune response is characterized by the secretion of cytokines (IL-4, IL-10, transforming growth factor [TGF]-β1) that modulate the cellular immune response.14,15 Finally, antigen can be presented and processed in such manner to induce regulatory T cells; these cells exert an immunomodulatory effect in response to antigen exposure and prevent activation of lymphocytes.16 These sce-
Because the BBB is disrupted after stroke, the immune system comes into contact with CNS antigens, in both the brain and periphery. We chose to exploit this fact and induce clones of regulatory T cells to myelin basic protein (MBP) that could potentially limit the inflammatory response after stroke. The regulatory T cells were generated according to the paradigm of mucosal tolerance before the ischemic insult.17 Animals were tolerized to MBP or ovalbumin (OVA), an irrelevant antigen, before 3 hours of middle cerebral artery occlusion (MCAO). Infarct size was measured either 24 or 96 hours after MCAO and was found to be less in animals tolerized to MBP.18 Immunocytochemistry revealed that lymphocytes infiltrating the infarct appeared to be producing TGF-$\beta_1$, implying a role for this cytokine in the neuroprotective effects of tolerance.18 Subsequent studies replicated these findings and showed that the neuroprotective benefits of the tolerance could be transferred to naive animals through systemic injection of tolerized lymphocytes at the time of reperfusion.19 That regulatory T cells are involved in this neuroprotection is implied by the fact that the cells from MBP-tolerized animals homed to the ischemic brain of naive animals and appeared to be secreting TGF-$\beta_1$; cells from OVA-tolerized animals did neither.19 Furthermore, ELISPOT assay showed that in comparison with OVA-tolerized animals, more lymphocytes isolated from the brains of MBP-tolerized animals secreted TGF-$\beta_1$ in response to stimulation with MBP; this increase in MBP-specific TGF-$\beta_1$–secreting cells was found in the brains of both sham-operated and ischemic animals.19 Subsequent studies have shown similar benefits by inducing tolerance to other CNS antigens in other animal models of cerebral ischemia.20–23 These experiments showed that because CNS antigens are exposed to the immune system after stroke, regulatory T cells tolerized to these antigens can be used to downregulate the inflammatory response in a “bystander” fashion.24

By tolerizing animals to MBP before MCAO, the immune system was educated to CNS antigens before the breakdown of the BBB. Because CNS antigens are exposed to the immune system after disruption of the BBB, however, the potential for developing an autoimmune response to the brain exists in immunologically naive animals (and people). We investigated whether or not such an autoimmune response after stroke exists by assessing the adaptive immune response to MBP. After 3 hours of MCAO, rats underwent varying periods of reperfusion. Mononuclear cells (MNCs) were isolated from the brain to characterize their immune response to MBP with ELISPOT assays; IFN-$\gamma$ was used to indicate a Th1 response (sensitization) and TGF-$\beta_1$, to indicate a Th2/Th3 response (tolerization/regulatory response). The difference in the number of cells secreting the cytokine in response to stimulation with MBP and the number that secreted the cytokine spontaneously was deemed to be the MBP-specific response. Animals with a ratio of MBP-specific IFN to TGF-secreting cells $\geq 2$ were considered to be “sensitized” to MBP; animals with a ratio of MBP-specific TGF to IFN-secreting cells $\geq 2$ were considered to “tolerized” to MBP. Up to 1 month after MCAO, there was little evidence that animals developed a Th1 response to MBP; on the contrary, the predominant response at 1 month was that of tolerance (Figure 1). A potential explanation for this type of response is suggested by the fact that autopsy and experimen-

**Figure 1.** a, There is no increase in the number of MBP-reactive cells secreting IFN-$\gamma$ relative to those secreting TGF-$\beta_1$ (ie, Th1 response) in animals 1 month after MCAO. b, In fact, there is a propensity for animals to become “tolerized” to MBP (an increase in the relative number of cells secreting TGF-$\beta_1$ to those secreting IFN-$\gamma$ when stimulated with MBP). *$P<0.05$, t test.
tal studies show that the normal and infarcted brain expresses a paucity of the costimulatory molecule B7.1; in fact, there is expression of a B7.1 homolog (B7-1H) that serves to inhibit the development of an adaptive immune response.25–27 Similarly, using immunocytochemistry, we found scant expression of B7.1 in either the normal or ischemic brain.28

Stimulation of TLRs is known to increase the expression of costimulatory molecules, including B7.1, on MNCs and microglia.29–35 Lipopolysaccharide (LPS), a component of the Gram-negative bacterial cell wall, is a potent agonist of TLR4; LPS is often used to stimulate the innate immune response and to model infectious insults. Infection is common after stroke, and occurrence of an infection in the immediate poststroke period is associated with a worse outcome.2,36–38 As it turns out, the predominant organisms causing infection after stroke are Gram-negative bacteria.39,40 There are several plausible mechanisms by which infection could worsen ischemic brain injury, but definitive mechanistic data are lacking. We explored some of the possible mechanisms by modeling the systemic inflammatory response that accompanies infection in our model of MCAO. In these experiments, a subset of animals received an intraperitoneal injection of LPS (1 mg/kg) at the time of reperfusion and underwent extensive immunological and histological analysis 1 month later.28 At this time point, there was a significant decrease in the size of the spleen and the number of splenocytes present in non-LPS, henceforth referred to as LPS(−), −treated animals; this decrease was not significant in LPS-treated animals (Figure 2a). There was also a decrease in the number of MNCs infiltrating the right/ischemic hemisphere of the brain in LPS(+) and LPS(−) animals 1 month after MCAO (Figure 2b), likely reflecting the amount of tissue that was destroyed by the stroke. Despite similar numbers of MNCs in LPS(+) and LPS(−) animals at this time point after MCAO, the phenotype of these cells differed, as evidenced by the fact that 67% of LPS(+) animals developed a Th1 autoimmune response to MBP, whereas only 22% of LPS(−) animals developed a similar response. As previously shown, administration of LPS was associated with increased expression of B7.1 early after stroke onset; there was also increased expression of vascular cell adhesion molecule-1 72 hours after MCAO in LPS(+) animals (P<0.05) (Figure 3). In addition, LPS(+) animals had more atrophy of the ischemic hemisphere 1 month after MCAO; these animals also had more CD8(+) cells in the brain and greater numbers of apoptotic neurons.28 CD8(+) cells are the effector cells of the immune system; they may kill their targets through induction of apoptosis or cytolysis (using the perforin/granzyme pathway).41–43 Both mechanism of cell death could occur in the CNS.44–48

Importantly, our experimental data suggest that development of a Th1 response to MBP is associated with worse neurological outcome after stroke. Animals that developed a Th1 response to MBP had worse neurological scores, performed more poorly on the sticky tape test, and did not gain weight as quickly as animals that did not develop a Th1 response to MBP.28 Whether similar deleterious autoimmune responses to the brain occur in people who experience stroke is unknown, but CNS autoreactive cells and CNS-specific immunoglobulins are seen in individuals with a history of...
cerebral ischemia. The long-term clinical consequences of this CNS autoimmune response are unknown, although there are several situations in which an autoimmune response to the brain could contribute to morbidity. For instance, autoreactive T cells could transit into the brain and enhance cerebral ischemic injury in patients who experience recurrent strokes. This potential is illustrated by the fact that mortality is greater after MCAO in rats immunized to MBP. Simi-

Figure 3. There is increased vascular cell adhesion molecule-1 expression after MCAO; the magnitude of the increase is greater in LPS(+) animals 72 hours after MCAO. *P<0.05, t test.

Figure 4. An overview of the possible immunological responses to brain antigens after stroke. Under normal circumstances, brain antigens do not enter the systemic circulation and lymphocytes do not enter the brain. After stroke, however, the encounter of CNS antigens by lymphocytes, in either the brain or systemic lymphoid tissue, leads to a Th2/Th3 immune response. If there is an infection after stroke, the concomitant inflammatory response may lead to changes in the microenvironment of the brain and systemic lymphoid tissue (such as expression of costimulatory molecules, like B7.1, and major histocompatibility complex antigens). These changes may predispose lymphocytes to develop a Th1 response to CNS antigens.
larly, transgenic animals with T cells that express receptors for MBP recover less well after spinal cord injury than do wild-type animals.54

Emerging data suggest that stroke induces an immunodeficiency that predisposes to infection.55–57 Teleologically, our data would suggest that this immunodeficiency prevents one from developing a deleterious autoimmune response to the brain. On the other hand, infection clearly can be fatal but potentially prevented by prophylactic antibiotics.56 Those patients who survive their stroke and infection, however, still evidence more disability than patients who do not develop infection. Our data demonstrate that a systemic inflammatory response can alter the microenvironment of the brain and support the development of an adaptive immune response to brain antigens after stroke.58 An overview of this hypothesis is illustrated in Figure 4. These findings may explain, at least in part, why infection in the immediate poststroke period contributes to worse outcome. We have also shown that induction of MBP-specific regulatory T cells is neuroprotective in the setting of stroke.18,19 Manipulating the immune response to brain antigens after stroke could therefore be of therapeutic value in preventing the postischemic autoimmune response. Experiments are currently under way to test this hypothesis.

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Disclosures
None.

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