Systemic Immune Deviation in the Brain That Does Not Depend on the Integrity of the Blood-Brain Barrier

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OVA injected into the brain of normal mice evoked a deviant immune response (brain-associated immune deviation (BRAID)) that was deficient in OVA-specific delayed-type hypersensitivity. This response was not dependent on an intact blood-brain barrier since BRAID was induced even when OVA was injected into a newly created lesion site with extensive BBB leakage. However, newly activated microglia at the injection site 2 days after ablation of the striatum correlated with the loss of BRAID. At day 4 after trauma, when activated microglia were only visible further away from the injection site, BRAID was again able to be induced. In contrast to immune deviation elicited via the eye, an intact spleen was not required for BRAID, nor was BRAID adoptively transferable with spleen cells. In contrast i.v. injection of cervical lymph node cells harvested 8 days after OVA injection into the striatum was able to transfer BRAID into naive animals. Together, these data indicate that immune privilege in the brain is actively maintained and is mediated by an immune deviation mechanism that differs from eye-derived immune deviation and arises even when the BBB is compromised. The Journal of Immunology, 2000, 164: 5125–5131.

The CNS has been shown to be an immune privileged site where transplanted allogeneic tissue can survive for a prolonged period of time (1–4). The classical description of immune privilege in the CNS invoked “immunological ignorance” as an explanation for prolonged survival of grafted tissue vis-à-vis conventional nonprivileged sites (1). Recent studies, however, have shown that Ag injected into the CNS is able to leave the brain and gain access to the systemic immune system. The main routes for drainage out of the CNS follow 1) the cerebrospinal fluid across the arachnoid villi into the blood and 2) the lymphatic pathway following the olfactory nerves into the cervical lymph nodes (5, 6). Drainage of CNS-derived Ag into the cervical lymph nodes leads to local production of Ag-specific Abs (6–8). In fact, Ab production is enhanced after CNS injection of Ag when compared with Ag injections into a nonprivileged site such as the nasal mucosa or the footpad (7). Interestingly, the enhanced Ab production does not lead to a rapid destruction of antigenic material in the CNS. There are at least two possible explanations for the enhanced survival of transplanted tissue in the CNS in spite of systemic recognition of the Ags. First, the immunological effector phase may be curtailed through the blood-brain barrier (BBB) and/or by down-regulation of effector cells and molecules once they penetrate the CNS. Second, Ags introduced into the CNS may evoke a deviant systemic immune response, one that leads to suppression of normal T cell effector activity.

To explore the second explanation, it is useful to examine another immune privileged site, the anterior chamber of the eye. Immune privilege and immune deviation in the eye have been extensively studied. Evidence derived from recent studies has revealed that immune privilege in the eye results from an active down-regulation of systemic and local immunity rather than passive “ignorance” (9–15). After injection of soluble Ag into the anterior chamber of the eye, local F4/80 APC leave the eye, enter the blood stream, and carry an immunological signal to the spleen (14). Within 1 wk, regulatory T cells emerge in the spleen and create Ag-specific suppression of delayed-type hypersensitivity (DTH) (9, 10). This deviant immune response (termed anterior chamber-associated immune deviation (ACAI)) can be adoptively transferred to naive animals with an i.v. injection of spleen cells from donors that received an anterior chamber injection of Ag 7 days previously (10). Additional features of ACAID are the presence of primed Ag-specific cytotoxic T cells that fail to become activated inside the eye (12) and B cells that secrete complement-fixing rather than complement-fixing Abs (15).

Our overall goal is to reassess immune privilege in the CNS and to evaluate whether the CNS facilitates the induction of deviant immune responses after injection of soluble Ag into the striatum. Additionally, we wanted to determine whether the systemic immune response to CNS Ag resembles ACAID.

We have chosen to examine the striatum in large part because this region is the site in which neural transplants have been placed by others in an attempt to ameliorate symptoms of parkinsonism (16). Therefore, understanding the immunological status of this site under various conditions may help us better understand the variable survival of grafted tissue in these experiments.

Materials and Methods

Animals

Adult female BALB/c mice, aged 6–10 wk, were obtained from the animal facilities at the Schepens Eye Research Institute or from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained in a common room of a vivarium. Injections and ear measurements were conducted under anesthesia induced by i.p. injection of 0.075 mg/kg body weight ketamine (Ketalar; Parke Davis, Ann Arbor, MI) and 0.006 mg/kg body weight xylazine.
(xyla-ject, Phoenix Pharmaceuticals, St. Joseph, MO). Five mice were used for each group and experiments were repeated at least twice. All experimental procedures conformed to the National Institutes of Health and Harvard Medical School regulations on the use of animals in research.

**Injections into the striatum**

OVA (a heterologous protein Ag; Sigma, St. Louis, MO) was used as a soluble Ag and was injected stereotactically into the striatum of adult BALB/c mice. A small craniotomy was performed over the region to be injected. A total volume of 1 μl of OVA (100 μg PBS) was injected into the dorsal caudate nucleus (stereotaxic coordinates: 2.0 mm lateral, 3.5 mm deep, 0.5 mm caudal to Bregma) via a pulled (tip diameter, −10 μm) 6.66-μl Micropipette (Drummond Scientific, Broomall, PA). The micropipette was attached to a 50-μl Hamilton syringe with polyethylene tubing, and the syringe was placed into a Microdrive apparatus (L. S. Starrett, Athol, MA) to allow slow, controlled injections. In additional groups of mice, the striatum received a lesion with an unpulled micropipette (outside diameter, 1 mm) 0–4 days before Ag injection. Control experiments demonstrated that injections made into previously ablated animals were indeed placed into ablated regions (data not shown).

**Assessment of BBB integrity**

Animals received an i.v. injection of HRP (5% in Tris buffer (pH 9.0), 1 mg/g body weight, grade I; Boehringer Mannheim, Minneapolis, MN) and were then sacrificed 15 min later. The brains were fixed and processed for histology. HRP was developed with Hanks-Yates reagent, and control sections were stained with hematoxylin–eosin. The sections were viewed under light microscopy and examined for the presence of extravasated reaction product, indicative of a leaky BBB.

**Immunohistochemical study of CNS tissue**

CNS tissue removed after insertion of pulled or unpulled micropipettes was fixed in 4% paraformaldehyde and cryopreserved in 30% sucrose/PBS. Frozen sections were then cut at 30 μm on a sledge microtome. Sections were stained with the following reagents: anti-CD45, to identify bone marrow-derived cells (PharMingen, San Diego, CA); Griffonia simplicifolia (GS)–isolectin–biotin conjugate, to identify vascular endothelial cells and microglia (Sigma); and anti-glial fibrillary acidic protein, to identify astrocytes (Zymed, South San Francisco, CA). Secondary Abs conjugated to Cy3 or FITC (Jackson ImmunoResearch, West Grove, PA), or in the case of GS-isolectin, avidin-PE reagent, were used to visualize the location of primary Abs via fluorescence microscopy.

**Assay for DTH**

DTH was evaluated 14 days after OVA injection into the striatum. Seven days after injection of OVA into the CNS, animals were immunized s.c. with 100 μg OVA and CFA. Ear-swelling analysis was performed 7 days later. DTH was measured based on ear swelling, as described previously (13, 17). Briefly, 200 μg OVA in 10 μl was injected into the left ear pinnae of the mice. The right ear served as untreat ed control. Both ear pinnae were measured immediately before injection and 24 h later with an engineer’s caliper (outside diameter, 1 mm) 0–4 days later. Ear swelling responses were assessed 24 h later. Data are presented as mean ear swelling ± SEM. ***, p < 0.001; **, p < 0.01; *, p < 0.05.

**Results**

**Systemic immune response to OVA injected into the striatum**

Our first goal was to determine whether an injection of Ag into the striatum of normal mice would elicit a systemic DTH response. With the aid of a stereotactically guided pulled glass micropipette, OVA (100 μg/1 μl) or PBS was injected into the dorsal caudate nucleus of brains of adult naïve BALB/c mice. Seven days later, these mice, as well as normal BALB/c mice serving as positive controls, received s.c. immunization with OVA (100 μg) mixed with CFA. One week thereafter, the ear pinnae of these mice were challenged with OVA (200 μg), and ear-swelling responses were assessed 24 and 48 h later. The results of a representative experiment are displayed in Fig. 1. Positive control mice, and mice that were immunized with OVA after an injection of PBS into the striatum, mounted intense DTH responses. In contrast, mice pre-treated with OVA into the striatum mounted only feeble ear-swelling responses, almost as low as those of negative controls. The inability of mice exposed via the striatum to OVA before immunization with OVA to acquire DTH implies that systemic immune deviation was induced. For convenience of further discussion, we have used the term brain-associated immune deviation (BRAID).

**Impact of intrastral injection on integrity of BBB**

Injections placed in the dorsal caudate nucleus with the utmost care must necessarily be disruptive to the local microanatomy, raising the likelihood that the BBB is breached. We examined this possibility using immunohistochemistry with the HRP method and with Abs capable of detecting the presence of bone marrow-derived cells, activated microglia, and astrocytes. Panels of BALB/c mice received injections of PBS (1 μl) into the dorsal caudate nucleus. In some mice, the striatum was ablated with an unpulled micropipette to create an extensive breach in the BBB. Immediately after the insertion of the micropipette, as well as 2 and 4 days later, HRP (1 mg/5 g body weight) was injected i.v. into recipient mice. Fifteen minutes later, the mice were killed and their brains were removed and prepared for histologic analysis. Sections of the ablated and adjacent site were stained for HRP reactivity. A summary of the results of this experiment is presented in Fig. 2. Brains removed 15 min after intrastral injection revealed heavy staining with HRP at the lesion site (Fig. 2A), which also diffused into
adjacent regions of the brainstem (Fig. 2B). After 2 days, no leakage of reaction product was seen (Fig. 2, C and D), indicating that the BBB had repaired itself by this time point. Similarly, no leakage of reaction product was seen in the brains of animals examined 4 days after ablation (Fig. 2, E and F).

We obtained almost identical results with the CD45 Ab and the GS-isolectin reactions, confirming that the cells labeled in both cases were indeed microglia. There is of course some controversy regarding the distinction between resident microglia and peripheral macrophages, and we have made no attempt to distinguish between these populations, but have used the term “microglia” to describe these cells. CD45 labels all bone marrow-derived cells, whereas GS-isolectin specifically labels α-galactose moiety on the surface of microglia. The photomicrographs in all cases show CD45 immunostaining. This Ab had a lower background than GS-isolectin, but as described above, appeared to label the same population of cells. Although very low levels of CD45⁺ cells (data not shown) were found in control or 0 day postlesion striatum, the margins of the lesion site contained detectable microglial cells by day 2 (Fig. 3B). By day 4, the margins of the lesion site no longer showed these cells, whereas microglial cells were now identified peripheral to the lesion site in the adjacent areas of the striatum (Fig. 3C). These cells displayed positive staining with GS-isolectin and CD45, suggesting that they are microglia. The results of these experiments are summarized in Table I.

These results indicate that the BBB was breached immediately and selectively at the site of inoculation and that the barrier was restored rapidly within 2 days. Moreover, dendritic cells (presumably activated microglia) were evident within ablated sites at 2 days after injection. At 4 days after injection, similar microglial

FIGURE 2. Time course of BBB breakdown and repair following striatal lesions: At various times after ablation, animals received a systemic injection of HRP (5 mg/kg), after which brains were fixed, removed, sectioned, and stained for HRP reaction product (Hanker-Yates reaction). 0 day: A and B. Immediately after lesion, the injection site shows widespread HRP reaction product in the parenchyma of striatum (A). This reaction product diffuses far into the adjacent parenchyma, distal to lesion cavity (B). 2 day: B and C. No leakage of HRP is detected at (B) or adjacent to (C) the lesion site. 4 day: D and E. As was seen at 2 days after ablation, no reaction product is detected at D or adjacent to E the lesion site.
cells were no longer evident within the lesion, but were clearly evident at the periphery.

Systemic immune response to OVA injected into previously ablated striatum

The tempo of alterations in the BBB and the tempo of changes in activated bone marrow-derived cells in and around the ablated site were disparate. We next examined the extent to which injection of OVA into ablated sites immediately after lesion placement would result in systemic immune deviation. Accordingly, lesions were created in the dorsal caudate nucleus of normal BALB/c mice with a blunt micropipette, as for the above described BBB assessment. In one panel of mice, a pulled micropipette was used to place a lesion in the dorsal caudate nucleus (minimal lesion). OVA was then injected into the ablated site immediately thereafter or at 2 or 4 days later. As before, the mice received an immunizing dose of OVA s.c. 1 wk later and were then ear challenged with OVA 7 days thereafter. As the results displayed in Fig. 4 indicate, reduced ear-swelling responses, compared with positive controls, were observed among mice that received intrastriatal OVA immediately after lesion placement, as well as 4 days after lesion placement. In contrast, mice that received intrastriatal injection of OVA 2 days after lesion placement developed vigorous ear-swelling responses. These results indicate that the presence of activated microglial cells at the OVA injection site, rather than integrity of the BBB at that site, correlates positively with the loss of the ablated site’s ability to promote immune deviation.

Influence of the spleen on BRAID

Injection of Ag into the anterior chamber produces a deviant systemic immune response similar to our findings with Ag injected into the striatum. The immune deviation associated with anterior chamber injections has been found to depend on the presence of an intact spleen. Anterior chamber injection of Ag into mice without spleens fails to induce immune deviation (11). To determine whether a similar situation applies to the brain, panels of BALB/c mice were splenectomized or sham operated 1 wk before OVA injection. The results displayed in Fig. 4 indicate, reduced ear-swelling responses, compared with positive controls, were observed among mice that received intrastriatal OVA immediately after lesion placement, as well as 4 days after lesion placement. In contrast, mice that received intrastriatal injection of OVA 2 days after lesion placement developed vigorous ear-swelling responses. These results indicate that the presence of activated microglial cells at the OVA injection site, rather than integrity of the BBB at that site, correlates positively with the loss of the ablated site’s ability to promote immune deviation.

Table I. Summary of experimental results

<table>
<thead>
<tr>
<th>Days Postlesion</th>
<th>BBB Integrity</th>
<th>Local Microglial Activation</th>
<th>BRAID</th>
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*This table highlights the importance of local microglial activation in the generation of a deviant immune response. In contrast, the presence of an intact BBB is not required for the induction of BRAID. BRAID was abolished by microglial activation, not BBB breakdown.*
was injected into the dorsal caudate nucleus. The mice were immunized subsequently with OVA plus CFA and ear challenged, as described above. Both mice with intact spleens and mice whose spleens had been extirpated 1 wk before intrastriatal injection of OVA mounted feeble ear-swelling responses. This result indicates that, unlike ACAID, the immune deviation that follows intrastriatal injection of Ag has no requirement for an intact spleen (Fig. 5).

Adoptive transfer of immune deviation with cervical lymph node cells

In ACAID, failed DTH is mediated in part by regulatory T cells of splenic origin. Cells of this type can adoptively transfer impaired DTH when injected into naive recipients. We wished to determine whether intrastriatal injection of Ag also generated regulatory T cells. The finding that splenectomy failed to promote DTH in mice that received an intrastriatal injection of OVA lessened the likelihood that regulatory T cells would be present in spleens of recipients of intrastriatal OVA. It has been previously demonstrated that Ags injected into the brain and CSF drain into lymphatic channels that lead to the cervical lymph nodes (6). This raised in our minds the possibility that the cervical lymph nodes might be a source of regulatory T cells. Accordingly, panels of BALB/c mice received injections of OVA into the dorsal caudate nucleus. Eight days later, the mice were killed, and their spleens and cervical lymph nodes were removed separately. Single-cell suspensions (6 × 10⁷ per recipient) from spleen or from lymph nodes were injected i.v. into naive BALB/c mice. Twenty-four h later, these mice were immunized s.c. with OVA plus CFA, and their ear pinnae were challenged with OVA 1 wk later. The results of representative experiments are presented in Figs. 6 and 7. Mice that received spleen cells from donors into whose striatum OVA had been injected displayed intense ear swelling similar to that of positive controls (Fig. 6). In contrast, mice that received cervical lymph nodes from similar donors displayed only feeble ear-swelling responses (Fig. 7). These results indicate that OVA injected into the striatum induced a population of regulatory lymphoid cells that emerged in the draining cervical lymph nodes. Cells of this type suppressed the development of DTH to OVA in recipients.
this protocol on the assumption that a simple microinjection of Ag into the striatum would not induce a deviant immune response. However, direct injection of soluble Ag (OVA) into the striatum in our experiments led to a systemic suppression of Ag-specific DTH, prompting us to follow a different experimental path.

An obvious methodological concern in these experiments involves the leakage of soluble Ag from the site of the injection. It is clear that regardless of the experimental procedure, Ag injected into the brain will rapidly drain to the cerebral lymph nodes (5). Similarly, injections made into the CNS of animals with “intact BBB” will cause a temporary breakdown of the BBB and subsequent leakage of Ag into the blood, which has previously been shown to cause an altered DTH response (19). However, if this was the sole cause of the diminished DTH response in these experiments, one would expect that injection of Ag into the brain would induce the suppression of DTH. In fact, at 2 days postablation, no suppression of DTH was observed. Moreover, the strongest suppression of DTH occurred not when widespread BBB leakage was present (day 0), but at day 4, when the BBB was reformed, and without the use of prior BBB disrupting lesion (minimal trauma; see Fig. 4). These results suggest that the prime factor that determines the immunological response to Ag in the CNS, as well as the eye, is not the presence or absence of a blood-tissue barrier or the amount of Ag “leakage”, but rather the environment in which initial Ag presentation takes place. This is supported by previous work in the eye, in which ACAID can be induced in spite of leakage of injected Ag or cells along the injection tract (10). Our results support the hypothesis that the local microenvironment is the strongest determinant of the immunological state of the CNS.

Induction of a deviant immune response to CNS Ag injection was not dependent on an intact BBB since BRAID was elicited when OVA was injected into a newly created lesion site with extensive BBB leakage. Surprisingly, BRAID was not induced when OVA was injected into a site ablated 2 days previously. Our observation that 2-day-old striatal lesions contain large numbers of activated microglia suggests that these cells may interfere with immune deviation. In support of this hypothesis is the finding that BRAID was induced if Ag was injected into a striatal site ablated 4 days previously; at this time, activated microglia were no longer prominent at the lesion, although significant numbers of such cells were observed in brain tissue surrounding the lesion. We suspect that breakdown of the BBB after initial trauma results in activation of resident microglia, perhaps under the influence of serum proteins, and that activated microglia prevent the induction of BRAID. Recent reports associate the presence of activated microglia with a higher incidence of graft rejection in the CNS (20). In our model, it is not yet clear whether the presence of microglia alone abolishes the induction of BRAID or whether the activation of microglia under the altered conditions of a breached BBB is responsible for the loss of BRAID. Further investigations are under way to evaluate the role of microglia in Ag presentation in the CNS under physiological and diseased conditions.

Our results highlight an important quandary regarding the route that Ags take to leave the CNS and the part microglia play in the presentation of those Ags to the systemic immune system. In the eye, another immune privileged site, there is circumstantial evidence that resident F4/80 APC pick up Ag under the influence of the unique ocular microenvironment. These cells acquire the ability to induce systemic immune deviation, which they accomplish by migrating across the trabecular meshwork directly into the blood (14).

Many authors have described the brain as an immune privileged site, where transplanted tissues show a prolonged survival (1–4). High Ab synthesis by both the cervical lymph nodes and the spleen have also been described as systemic characteristics of a deviant immune response elicited in the brain (6–8). Our results indicate that injection of soluble Ag into the striatum leads to a suppression of Ag-specific DTH similar to that described for ACAID (9). In ACAID, regulatory T cells have been found to be responsible for the suppression of Ag-specific DTH (10). These T cells emerge from the spleen, the primary draining lymphoid organ of the eye. In contrast to the immune deviation elicited in the eye (10, 13), an intact spleen was not required for BRAID, since BRAID could be induced in splenectomized mice. Additionally, we were not able to adoptively transfer BRAID into naive mice by spleen cells harvested after injection into the striatum. This indicates that suppression of DTH in BRAID does not follow the same splenic pathway as described for ACAID.

One of the immunological differences between the eye and the brain is the amount of lymphatic drainage out of each organ. Aqueous humor drains mainly via the trabecular meshwork into the venous system and only a very small portion leaves the eye via the uveoscleral route. Therefore, not more than 10% of protein injected into the eye ends up in the draining lymph nodes. The situation in the brain is probably different, because 14–47% of injected protein drains into the lymphatic system (5). The remaining protein leaves the brain with the cerebrospinal fluid through the arachnoid villi into the blood.

One report suggests that draining cervical lymph nodes are involved in an immune response elicited in the brain, stating that there is B cell expansion in the cervical lymph nodes after Ag injection into the brain (6). The Ab production is significantly increased compared with injections into other body sites (7).

We tested the influence of cervical lymph nodes on the suppression of DTH after OVA injection into the striatum. We harvested these nodes and reinjected the cells into naive animals. In contrast to similar experiments performed with spleen cells, cervical lymph node cells of mice following an OVA injection into the striatum were able to transfer the deviant immune response to naive mice. This result demonstrates that cells within the draining cervical lymph nodes possess the ability to suppress Ag-specific DTH. In ACAID, the regulatory cells are derived from the spleen and have been found to be to be CD8+ and Thy1.2+ T cells. The cells responsible for the deviant immune response elicited in the CNS remain to be identified and characterized.

Our data indicate that immune privilege in the brain is actively maintained. It exists even when the BBB is compromised, but is abolished at a lesion site with high local levels of microglial activation. BRAID is mediated by an immune deviation mechanism that differs from ACAID because an intact spleen is not required and can be adoptively transferred by cells isolated from cervical lymph nodes. Furthermore, these results suggest that studies in which tissue is transplanted into the CNS should take account of the importance of resident APC at the transplantation site in the pattern of the immune response generated to foreign Ags injected into the CNS.

Acknowledgments

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