Antigen-Dependent Intrathecal Antibody Synthesis in the Normal Rat Brain: Tissue Entry and Local Retention of Antigen-Specific B Cells


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Antigen-Dependent Intrathecal Antibody Synthesis in the Normal Rat Brain: Tissue Entry and Local Retention of Antigen-Specific B Cells

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The intrathecal Ab response to Ag introduced into the normal brain has not been fully explored. Involvement of Ag-specific, peripheral B cells in an intrathecal response was studied using a normal rat model of Ag infusion through an indwelling cannula into defined brain sites, while maintaining a functionally intact blood-brain barrier. Specific Ab was detected in serum and cerebrospinal fluid. The intrathecal response is first detectable at day 14. Isoelectric focusing of cerebrospinal fluid reveals banding patterns consistent with local Ab production. To increase Ag-specific, circulating peripheral lymphocytes available for trafficking to Ag-stimulated brain and for enhancing intrathecal Ab synthesis, rats were preimmunized peripherally. Subsequently, Ag or saline (control) was infused through the cannula. Under this protocol, intrathecal synthesis is detectable earlier (day 5 postinfusion). Immunohistochemical studies at the infusion site assessed Ag-specific B cells, T cells, and activated APCs. Rats receiving peripheral preimmunization followed by Ag into caudate nucleus have far greater numbers of these cells, including plasma cells, within the infusion site compared with saline controls. Results confirm previous indirect evidence of intrathecal Ab synthesis in normal rat brain and provide the first direct evidence for B cell trafficking across normal brain barriers plus retention at the Ag deposition site. Our studies indicate that the normal brain microenvironment supports development of Ag-directed humoral immunity. We propose that immune privilege in normal brain is characterized by down-regulation of cell-mediated but not Ab immune responses within the central nervous system. The Journal of Immunology, 1998, 161: 692–701.

The presence of lymphocytes in the central nervous system (CNS)3 or of Abs in cerebrospinal fluid (CSF) is generally associated with disease states, such as meningitis and encephalitis, in which the normal functions of the blood-brain barrier (BBB) have been compromised (1). Likewise, although the etiologies of autoimmune neurologic diseases, such as multiple sclerosis, are not fully understood, the presence of CSF Abs in such cases are generally attributed to local intrathecal synthesis by oligoclonal, Ag-specific B cells infiltrating an already damaged BBB.

In contrast, previous work of Cserr and Knopf (2, 3) suggests that intrathecal Ab synthesis within the brain may not just be a sequela associated with CNS diseases involving a compromised BBB; it can be elicited in a healthy animal with normal BBB permeability. A model was developed to study regulation of immune responses in the brain of normal rats (4). In this model, a small volume of Ag in saline is slowly microinfused through an indwelling cannula localized to a specific region of the brain by stereotactic placement. Ag infusion is conducted 1 wk following cannula implantation to allow the BBB to regain normal permeability (5). Specific Ab tiers can be detected in serum within 1 wk postinfusion and persist for several months. In addition, specific Ab can be detected in CSF at 3 wk postinfusion, at levels in excess of values predicted by passive influx from plasma, via a normal leakage pathway determined by molecular size (6). In these rats, normal barrier permeability was confirmed by measuring relative concentrations of endogenous albumin levels in CSF and serum. Moreover, excess CSF Abs were never detected in rats peripherally immunized with Ag plus adjuvant, even when serum Ab titers generated by this hyperimmunization protocol were up to two orders of magnitude higher (6). Specialized Ig transport into the CNS has also been excluded (7). We have therefore concluded that intrathecal synthesis is required to account for the excess of specific Ab in the CNS following Ag stimulation to the brain.

Evidence for a humoral immune response within an Ag-stimulated but apparently healthy brain is in contrast to the immune privilege status of this organ with respect to allogeneic tumors and tissue transplants and their prolonged survival in normal brain (2). Historically, the survival of these tissues was attributed to an apparent lack of an effective immune response within the normal brain, which was a consequence of two unique anatomical features: the absence of classic lymphatic drainage within the nervous tissue and the presence of the BBB. Together, these features could block afferent and efferent arms of an immune response to Ag introduced into the brain. It is now clear that interactions do occur between the normal CNS and immune system regardless of these features (2, 3). Immune responses within the brain are regulated...
and are not a passive consequence of anatomical isolation, although a regulatory role for the barriers is probable (8–11). Furthermore, a modern model for CNS immunity cannot assume that humoral and cell-mediated arms of the immune response are regulated in the same manner. In our normal rat brain model, CSF Abs were elicited by introducing T-dependent Ags into the brain, indicating that interaction between Ag-specific B and T cells, together with Ag (native and processed, respectively), must have occurred at some point. It is known that activated T cells can enter the normal CNS regardless of Ag specificity or MHC compatibility with the host; retention of activated T cells within the brain required the MHC-restricted recognition of specific Ag (8, 9). However, little has been done to assess the competency of activated B cells to cross into the normal brain.

In the present study, three series of experiments were performed to further characterize the intrathecal Ab response, using our normal rat brain model for placing Ag behind an intact BBB (12). First, the kinetics of intrathecal Ab synthesis was established following a single infusion of Ag into CSF through an indwelling cannula, the previously published model. CSF and serum Abs were also characterized by isoelectric focusing (IEF) and immunoblotting to immobilized Ag. In the latter two series of experiments, the protocol was modified to increase the numbers of circulating Ag-specific lymphocytes, by i.m. immunization with Ag plus CFA. Subsequently, Ag or saline (control) was infused through the indwelling cannula. In addition to determining the kinetics of the intrathecal response and characterizing specific Abs by immunoblotting, the cannula was placed into the brain parenchyma (caudate nucleus) to facilitate immunohistochemical analysis at the site of Ag deposition. The presence of Ag-specific B cells and CD4+ T cells and the up-regulation of MHC class II expression at the site of Ag infusion were assessed. The results of this study support the hypothesis that B cells have the ability to seek Ag, even behind the BBB, and to differentiate into plasma cells in the tissue in which the Ag is encountered.

Materials and Methods

Animals

All experiments were performed using male, viral Ag-free rats (either Sprague Dawley from Charles River Breeding Laboratories, Wilmington, MA, or Lewis, from National Cancer Institute, Bethesda, MD), weighing 150 to 400 g at time of cannula implantation. Anesthesia for surgical implantation was achieved using a mixture of ketamine HCl (60 mg/kg; Aveco, Fort Dodge, IA) and xylazine (35 mg/kg; Rugby Laboratories, West Chester, PA) injected i.m. (1 mg HSA., day –7, after completion of implantation surgery); HSA (90 µg in 9 µl saline) and saline (9 µl) infused on day 0; Sprague Dawley rats sacrificed on either day 5 (n = 5) or day 14 (n = 6) postinjection. Serum and CSF were analyzed for anti-HSA and RSA and for oligoclonal banding by IEF and immunoblotting, as described below.

Protocol IIA: cannula was implanted in caudate nucleus (d. –7); OVA in CFA injected i.m. (1 mg OVA, day –7, after completion of implantation surgery); OVA (45 µg in 0.75 µl saline, n = 4) or saline (0.75 µl, n = 2) infused on day 0; Lewis rats sacrificed on day 14. Serum and CSF were analyzed for anti-OVA and RSA. Brains were fixed and removed for histology, as described below.

Protocol IIB: cannula was implanted in caudate nucleus (d. –40); OVA in CFA injected i.m. (1 mg OVA, day –40, after completion of implantation surgery), and again on day –7; OVA (45 µg in 0.75 µl saline, n = 4) or saline (0.75 µl, n = 2) infused on day 0; Lewis rats sacrificed on day 9. Serum and CSF were analyzed for anti-OVA and RSA. Brains were fixed and removed for histology, as described below.

Assays

Blood was removed either from the tail vein or, in more recent experiments, by cardiac puncture. After clotting, serum was collected by centrifugation and frozen at –70°C until assay. CSF was withdrawn from the cisterna magna as previously described (6). An ELISA was used to measure anti-HSA or anti-OVA binding to immobilized Ag and for RSA detection using immobilized anti-RSA (6). The Ab quotient (QAb) was calculated as the ratio of CSF Ab titer × 1000 to serum Ab titer. The RSA quotient (QRSa) was calculated using CSF and serum concentrations of this protein × 1000. The Ab index (IAb) was calculated as (QAb)/(QRSa), normalized for the typical barrier leakage of protein from blood to CSF (13). The QRSa is a measure of passive “leakage” or permeability of brain barriers to RSA, a protein plasma synthesized in the liver and not the brain. The QAb is a measure of barrier leakage to plasma Abs plus excess Ab from other sources (e.g., intrathecal synthesis, receptor-mediated transport). In animals not immunized in the CNS, QAB is less than QRSA, since normal barrier permeability is inversely proportional to protein size, and IAb is <1.0 (range 0.3–0.8 in our laboratory). If the IAb in CNS-immunized animals exceeds this range, the excess Ab is attributed to intrathecal synthesis, since we are unable to demonstrate receptor-mediated transport in this system (7). This procedure for determining intrathecal Ab synthesis is standard in the nervous immunology literature.

Immunoblotting to detect specific Ab or total Ig was conducted on serum and CSF samples following IEF on agarose slab gels as previously described (14). The sample volumes loaded on gels are described in the figure legends. After electrophoresis for 1000 V-h, one-half of the gel was rinsed in saline, and immersed in 50 ml of 0.1% milk/saline containing 50 µl 0.5% HSA in saline, 0.05% sodium azide, followed by blocking in 1% skim milk. The duplicate half of the gel was blotted to a nitrocellulose membrane. The membranes were then blocked in 1% skim milk in saline, rinsed in saline, and immersed in 50 ml of 0.1% milk/saline containing 50 µl peroxidase-conjugated anti-rat γ-globulins (Dako, High Wycombe, U.K.) and incubated at room temperature overnight with rocking. The membranes were thoroughly washed in tap water and saline, then developed using ethylamino carbazole and hydrogen peroxide.

Immunohistochemistry

Rats under deep anesthesia were perfused via the aorta with 50 ml of Dulbecco’s modified saline followed by 200 ml of 4.0% paraformaldehyde in 0.1 M phosphate buffer. pH 7.4. Following removal, the brain was sectioned in a horizontal plane and washed extensively in 0.2 M phosphate buffer, pH 7.4. Brain sections were equilibrated overnight with 30% sucrose for cryoprotection, and frozen in OCT mounting medium (Miles Laboratories, Elkhart, IN).

Cryostat sections (5 µm thick) were mounted on precleared glass slides, and immunohistochemical staining was performed, as extensively detailed previously (15). Primary murine mAbs used for tissue staining (Serotec, Washington, DC) were: OX-6 against rat MHC class II molecules, R7.3 specific for rat αβ TCRs, W3/25 anti-rat CD4, OX-8 anti-rat CD8, OX-42 against rat CD11b/c, and ED-2 identifying a specific subset of macrophages. In addition, OVA was conjugated to FITC for direct use as a fluorescent ligand specific for Abs and Ab-bearing cells reactive with OVA. Biotinylated anti-rat IgG (Vector Laboratories, Burlingame, CA) was used to identify rat plasma and B cells, and rat-absorbed biotinylated anti-murine IgG (Vector Laboratories) was used to localize the murine primary Ab. To localize the biotinylated Abs, either rhodamine-conjugated avidin (Sigma Chemical, St. Louis, MO) or peroxidase-conjugated avidin (Vector Laboratories) was employed.
A series of double-labeling studies was performed in which OVA was biotinylated and Abs against rat CD11b/c (OX-42) or rat MHC class II (OX-6) were coupled to FITC. A 1:1 mixture of biotinylated-OVA plus either FITC-OX-42 or FITC-OX-6 was layered over the tissue and incubated overnight at 4°C. Following rinsing of the slides, the tissue was exposed to streptavidin-conjugated Texas Red (Sigma) for 2 h at room temperature, rinsed, and coverslips mounted for viewing by confocal microscopy.

Results

Characterization of CSF anti-HSA Abs in rats receiving HSA by a single CSF infusion (protocol I)

In this first set of experiments, the appearance of Ag-specific Ab in CSF was characterized over a 3-wk period following a single infusion of HSA into the CSF (cannula in lateral ventricle) of Sprague Dawley rats (protocol I). Anti-HSA titers and levels of RSA in both CSF and serum were measured from individual rats at the different times postinfusion and used to calculate the IAb, which is a measure of the Ab titer in CSF normalized for protein leakage from blood to CSF (see Materials and Methods). For comparison, IAb data from two other conditions were analyzed: for rats immunized i.m. with HSA plus CFA but with no Ag infusion into CSF; and for nonimmunized (naive) rats in which total rat Ig levels measured in CSF and serum were used to calculate the index.

Figure 1A shows IAb values at several times, beginning on day 5, when serum anti-HSA titers are barely detectable, through day 21, when serum anti-HSA levels are approaching maximal values (Table I) (4). Anti-HSA is not detectable in CSF on day 5. By day 10, anti-HSA is detectable in only 1 of 8 rats, and the IAb value for that case is not greater than would be expected from passive leakage of serum Ab (Fig. 1A, NAIVE or IM values). Anti-HSA Abs in CSF are increased on day 14, and IAb values are greater than expected for passive leakage in two of five rats. On day 21, six of six rats had detectable anti-HSA Abs in CSF, and four of these had elevated IAb values, ranging from 1.1 to 16.8. The indices for the other rats were in the range of normal passive leakage (0.3–0.6). Having excluded damage to the BBB (QRSA is in the normal range, Table I) and excluding a mechanism of active Ig transport (IAb values of peripherally immunized rats is 0.3–0.6, Fig. 1A), these data indicate that intrathecal Ab synthesis contributes to the elevated anti-HSA Ab levels in CSF for rats receiving a single CSF infusion of the Ag. The intrathecal synthesis becomes detectable around 2 wk postinfusion.

Anti-HSA Abs in CSF and serum at day 21 postinfusion were further characterized by IEF plus immunoblotting (Fig. 2). IgG anti-HSA was detected in CSF with oligoclonal banding patterns in all cases. Diluting the serum to achieve anti-HSA titers in the same range as those in CSF, only two samples had significant IgG anti-HSA staining. This lower staining activity is attributed to the greater heterogeneity of anti-HSA in serum relative to CSF. The staining for total IgG in these samples (data not shown) showed sufficient intensity to be confident of visualizing Ag-specific Ab. The CSF findings are definitive examples of oligoclonal banding (16), except that in this model the rats are normal, i.e., without overt CNS pathology. These results further strengthen the conclusion that primary administration of Ag into CSF elicits a specific humoral immune response within the CNS.

Characterization of CSF anti-HSA Abs in rats receiving both a peripheral preimmunization and infusion of HSA into CSF (protocol II)

In previous work, we have shown that a single CSF infusion of HSA elicits Ag-specific Ab production in cervical lymph nodes and spleen, with the nodes being essential for induction of the response (4). This peripheral response may be the source of B cells generating intrathecal Ab synthesis in the experiment described above. To increase the number of HSA-specific lymphocytes available for trafficking to the brain and thus strengthening the intrathecal Ab response to CNS Ag, the experimental protocol was modified to expand the pool of HSA-specific lymphocytes circulating in blood. Rats were preimmunized with an i.m. injection of HSA in CFA 1 wk before CSF infusion of either HSA or saline (control). Recirculating lymphoblasts are expected to be at a high concentration at the time of CSF infusion (17). CSF and serum

Table I. Serum anti-HSA titers and QRSA following single infusion of HSA into CSF (no preimmunization)

<table>
<thead>
<tr>
<th>Day</th>
<th>Serum Anti-HSA</th>
<th>QRSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 5</td>
<td>&lt;10</td>
<td>Not determined</td>
</tr>
<tr>
<td>Day 10</td>
<td>615 ± 40 (40–4060) (10)</td>
<td>1.17 ± 0.26 [0.46–1.90] (5)</td>
</tr>
<tr>
<td>Day 14</td>
<td>1640 ± 150 (80–6060) (6)</td>
<td>2.09 ± 0.28 [1.67–2.62] (3)</td>
</tr>
<tr>
<td>Day 21</td>
<td>3050 ± 156 (670–8790) (6)</td>
<td>2.19 ± 0.19 [1.56–2.85] (6)</td>
</tr>
</tbody>
</table>

a Geometric means ± SEM and range in parentheses (n rats).
b RSA_{csf}/RSA_{serum} × 1000; arithmetic means ± SEM (n rats)
samples were collected on day 5 and 14 postinfusion and IAb values determined (Fig. 1B). In addition, CSF and serum samples were subjected to the IEF/immunoblotting procedure (Fig. 3; Table II).

For both day 5 and 14 postchallenge (Fig. 1B), the IAb values are greater in the preimmunized rats that received HSA by CSF infusion, compared with the saline infusion controls. On day 5, the IAb values are 2 to 4 times higher (HSA vs saline) and on day 14 are 2 to 20 times higher. These results are to be compared with the previous experiment (Fig. 1A) in which there was no detectable CSF Ab at day 5 and no significant IAb values above the normal range for passive leakage until day 14. The day 14 data in Figure 1B resemble the day 21 data in Figure 1A. Thus, peripheral preimmunization has significantly accelerated the onset of intrathecal Ab synthesis following the infusion of HSA into the CSF. It appears that the entry and/or retention of Ag-specific lymphocytes is enhanced within the CNS in this preimmunization model and is dependent upon infusion of Ag into CSF.

**FIGURE 2.** IEF/immunoblot analysis of CSF and serum (1:300) from Sprague Dawley rats immunized 21 days earlier (from Fig. 1A) via infusion of HSA through an indwelling intraventricular cannula. Electrophoretic patterns of anti-HSA were assessed by densitometric tracing. Prominent peaks representing high concentrations of anti-HSA from oligoclonal B cells are present in all CSF samples, but can be detected in only two serum samples, which is attributed to the more polyclonal distribution of the latter.
Confirmation of this result is obtained by analyzing CSF and serum using IEF/immunoblotting to immobilized Ag, HSA (Fig. 3; Table II). Comparing anti-HSA intensities of the undiluted CSF and diluted serum samples by densitometry, rats receiving an HSA infusion into CSF have higher Ab ratios (CSF/serum) relative to the saline-infused controls (Table II). These results confirm the data obtained using ELISA (Fig. 1B). Two of the three rats at day 5 and all three rats at day 14 post-CSF infusion of HSA have anti-HSA quotients that are 2- to more than 10-fold greater than the saline controls. The oligoclonal bands in CSF were less pronounced using this protocol, but an example is shown in Figure 3. Due to peripheral preimmunization with Ag in CFA, serum Ab titers are much higher and contribute to CSF Ab titers due to the normal passive leakage of plasma proteins into CSF. The QRSAs (Table II) from these samples, used to calculate IAb values in Figure 1B, establish that the brain barrier membranes are not compromised by the peripheral immunization with CFA present. These results confirm an earlier study using two different Ags for immunization simultaneously, OVA in CSF and HSA + CFA peripherally. IAb for anti-OVA was above IAb for anti-HSA in all cases, and the range of values for albumin quotients was within normal limits (6).

Thus, onset of intrathecal Ab synthesis can be accelerated in rats preimmunized with HSA + CFA in the periphery. Stimulation is dependent upon infusion of the preimmunizing Ag into the CSF through an indwelling cannula. Since B cells are the source of Ab-secreting plasma cells, such cells would be expected to increase in numbers within the brain of rats due to enhanced cell traffic to the brain or higher retention of Ag-reactive cells, or both.

Immunohistochemical analysis at the site of Ag deposition in rats receiving a systemic immunization of OVA plus adjuvant, followed by OVA infusion into caudate nucleus (protocols IIIA and IIIB)

To facilitate immunohistochemical analysis of the Ag-stimulated CNS, the infusion site for Ag was changed to the caudate nucleus, thereby localizing Ag to a specified region within the brain parenchyma. Rats were preimmunized at a peripheral site by i.m. injection with OVA plus CFA and subsequently received an infusion of OVA in saline via the indwelling cannula. Two control groups were also analyzed: one to determine the effects of an indwelling cannula with no peripheral or central exposure to Ag (negative control); the other to determine the effects of peripheral preimmunization only (saline-infused into cannula). In the latter control, no direct Ag stimulation was administered to the brain.

In the brains of negative control rats, scattered within the tissue lining the cannula track and the zone around the cannula tip, macrophages and rare CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes were found. Also in the vicinity of the cannula tip, there were reactive microglial cells expressing MHC class II and CD4 molecules, as would be expected around a cerebral stab wound in a rat after 2 wk (18).

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Table II. Ag-dependent intrathecal synthesis of specific Ab in rats receiving a peripheral preimmunization before Ag infusion

<table>
<thead>
<tr>
<th>Rat</th>
<th>Infusion</th>
<th>Assay (day)</th>
<th>Anti-HSA Density&lt;sup&gt;a&lt;/sup&gt; (CSF/serum)</th>
<th>QRSAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>Saline</td>
<td>5</td>
<td>0.25</td>
<td>3.4</td>
</tr>
<tr>
<td>2A</td>
<td>HSA</td>
<td>5</td>
<td>0.47</td>
<td>3.4</td>
</tr>
<tr>
<td>3A</td>
<td>HSA</td>
<td>5</td>
<td>1.17</td>
<td>3.4</td>
</tr>
<tr>
<td>5A</td>
<td>HSA</td>
<td>5</td>
<td>0.52</td>
<td>2.0</td>
</tr>
<tr>
<td>6A</td>
<td>Saline</td>
<td>14</td>
<td>2.21</td>
<td>2.0</td>
</tr>
<tr>
<td>3B</td>
<td>Saline</td>
<td>14</td>
<td>0.15</td>
<td>2.8</td>
</tr>
<tr>
<td>4B</td>
<td>HSA</td>
<td>14</td>
<td>0.14</td>
<td>1.1</td>
</tr>
<tr>
<td>6B</td>
<td>HSA</td>
<td>14</td>
<td>0.17</td>
<td>2.0</td>
</tr>
<tr>
<td>1B</td>
<td>HSA</td>
<td>14</td>
<td>1.33</td>
<td>3.4</td>
</tr>
<tr>
<td>2B</td>
<td>HSA</td>
<td>14</td>
<td>2.11</td>
<td>1.3</td>
</tr>
<tr>
<td>5B</td>
<td>Saline</td>
<td>14</td>
<td>2.00</td>
<td>1.0</td>
</tr>
</tbody>
</table>

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<sup>a</sup> Ratios of areas under profiles generated by densitometry tracings of anti-HSA separated by IEF (see Fig. 3, rats 2A and 3A, for typical result).
Immunohistochemical staining for the presence of rat IgG revealed only a modest blush that was closely confined to the region of the cannula (Fig. 4, top). There was no evidence of anti-OVA IgG, nor were there cells of macrophage or lymphocytic morphology that bound the FITC-OVA conjugate. Importantly, the presence of a caudate cannula resulted in minimal reaction on the part of the brain parenchyma, and there was no evidence of an anti-OVA response of any type (although macrophages phagocytosing cellular debris were present in the lumen of the cannula track). Since the negative controls were not immunized with OVA, these latter observations confirm the specificity of the FITC-OVA conjugate.

In many ways, the results observed in the brains of peripherally preimmunized control rats receiving saline intracranially via the cannula were similar to the negative controls in both versions of protocol III. Macrophages and T lymphocytes were also found scattered about the tissue lining the cannula track and in the lumen at the tip of the cannula. In this region, microglial cells were reactive and had become positive for MHC class II and CD4, differing from the negative control but significantly less positive than the OVA-infused preimmunized rats (see below). Staining for rat IgG again demonstrated a small blush confined to the immediate area of the cannula track, quite similar to that seen in the negative controls (Fig. 4, middle). Staining of the area with FITC-OVA revealed that positivity with this probe was coextensive with the staining for rat IgG, an expected result because normal leakage of the high titer anti-OVA from serum had occurred. Moreover, two types of cellular staining could be identified. Some cells showed a hazy, diffuse positivity of the cell membrane and granular positivity of the cytoplasm. By their location, number, morphology, and similarity to OX-42 staining (CD11b+) cells in other sections, it was possible to conclude that they represented perilesional macrophages that had become coated with anti-OVA Abs. In addition to these macrophages, there was an occasional round cell that had a rim of bright positivity and diffuse, less intense cytoplasmic staining with FITC-OVA. These cells were found around the cannula track and in the parenchyma, albeit they were few. It is presumed that they represent a lymphocyte population that is either B cell-specific for OVA or some other form of lymphocyte that had bound anti-OVA IgG to its membrane.

Double-labeling studies were then performed as a way of distinguishing OX-42 (anti-CD11b, CR3)-positive cells bearing FcR from B lymphocyte cell lineages. For this analysis, biotinylated OVA and FITC-OX-42 conjugates were placed on the same tissue section, with bound biotinylated-OVA detected by streptavidin-Texas Red. New sections were prepared for this study using confocal microscopy. Cells staining with single conjugate alone were not detected, confirming their rarity in preimmunized rats receiving saline through the cannula (Fig. 5). The only stained cells detected were brownish-orange to yellowish-orange in color, indicating colocalization of binding both the red and green conjugates, perhaps at differing ratios. Morphologically, these cells displayed the bound conjugates as discrete aggregates, as if they had collected within vesicles. We identify these cells as the perilesional macrophages described above.

Strikingly, the immunohistochemical results from rats that were both preimmunized peripherally and infused with Ag into the caudate are in sharp contrast to the above two categories of control rats. In the region of the caudate nucleus of these Ag twice-exposed animals—centered on but not limited to the cannula bed—macrophage/monocyte cells and lymphocytes had collected in high frequency. Both CD4+ and CD8+ lymphocyte phenotypes were present. Staining of the area for rat IgG revealed a wide zone of positivity, occasionally involving the entire hemisphere on the side of the brain with the cannula (Fig. 4, bottom). Staining of adjacent
sections with FITC-OVA also demonstrated a wide zone of positivity with this probe that corresponded to the area of IgG positivity. Inspection of the cellular components in the region lining the cannula track demonstrated the FITC-OVA-positive macrophages and B cells/lymphocytes noted above; yet they were both present in much higher numbers. Moreover, in the parenchyma around the cannula site, there was now a broad zone of microglial positivity for MHC class II and CD4, as well as enhanced prominence of astrocytes. In the parenchyma, lymphocytes and macrophages (or activated microglial cells) were present in notably greater numbers than in other groups of rats. In addition, it was possible to detect a type of cell with the FITC-OVA that had not been noted in prior groups of rats. These cells were encountered infrequently, but could occasionally be found singly or in small groups and occasionally in clusters of three or four around small blood vessels. They had a small round nucleus and abundant cytoplasm that stained brightly and homogeneously with FITC-OVA; these were judged to be OVA-specific plasma cells.

To confirm the presence of plasma cells in these twice-immunized rats, the double-staining technique described above was applied to new sections of the tissue samples (Fig. 6, upper and lower panels). In these sections, rats infused through the cannula with OVA 9 days previously (protocol IIIB) were used, since this was within the time period when intrathecal synthesis was not detectable in protocol I. Quite convincingly, individual cells staining one of three different colors were detected, which easily distinguishes them from the saline-infused, peripherally immunized controls described above. Cells staining bright green, with dendritic-like extension (CD11b+/anti-OVA−) were frequent and probably included the activated microglial population detected above (MHC class II+/CD4+). They were more extensive when Ag was present (compare with Fig. 5). Cells staining orange (CD11b+/anti-OVA−) with the vesicular morphology are likely to be the macrophages prominent in the saline-infused controls (Fig. 5). Cells staining red (CD11b−/anti-OVA−) with either a central nucleus or brighter red with an eccentric nucleus were also frequent. The former are presumed to be B cells displaying surface Ig with anti-OVA specificity. The latter are presumed to be plasma cells that have been sliced through during sectioning, allowing access of the biotinylated OVA to the cytoplasmic Ig destined for secretion. Thus, the presence of plasma cells and B cells in the brain parenchyma is a distinguishing and specific feature of the peripherally immunized rats that have received an OVA challenge into the brain parenchyma. Peripheral preimmunization accelerates the appearance of these B cells, which require Ag to be present in the
brain parenchyma to accumulate and possibly to differentiate. Since circulating plasma cells are rarely found, we presume that plasma cells found in the brain parenchyma were derived from the B cells present in a cytokine-dependent reaction.

**Discussion**

The results of this study provide firm evidence to support the inferences of our previous experiments (6): infusion of a T-dependent protein Ag into a brain with normal barrier permeability results in an intrathecal humoral immune response. Using immunohistochemistry, we have shown that OVA-binding CD11b+ cells are detected within Ag-infused (but not saline-infused) brains of rats peripherally preimmunized with OVA, a T-dependent Ag (Figs. 5 and 6). Morphologically, some of these Ag-binding cells satisfy the criteria that define B cells and/or activated B lymphocytes trafficking into the brain enter a microenvironment that can constitutively support B cell differentiation into Ab-secreting plasma cells. The importance of a peripheral source of Ag-activated lymphocytes for CNS humoral immunity was demonstrated by experiments characterizing the kinetics of IAb elevation in rats receiving a protein CSF infusion with or without peripheral preimmunization (Fig. 1, B and A, respectively). For rats receiving only a CSF infusion (protocol I), intrathecal synthesis did not precede the appearance of Abs in blood (Table I) and was only detectable beginning 2 wk post-CSF infusion of protein (Fig. 1A). These results suggest that, following Ag infusion, a period of about 14 days is required for a series of events to occur: efflux of Ag from brain to draining lymphoid organs, Ag-specific lymphocyte recruitment and expansion within these organs, trafficking of activated immune cells via blood to the brain, and their subsequent retention plus B cell differentiation to plasma cells. In contrast, peripheral preimmunization greatly reduced the time interval between Ag infusion into the brain and appearance of excess Abs in the CSF; in these rats, intrathecal synthesis was already detectable by 5 days post-CSF infusion (Figs. 1B and 3; Table II). Since Ag efflux from the CNS has been shown to occur relatively rapidly ($t_{1/2} = 12 h$), it seems most probable that the most time-consuming events for intrathecal synthesis to become established in the brain are related to peripheral induction of the humoral immune response, i.e., lymphocyte recruitment, expansion, and differentiation, which must occur first in draining lymphoid organs and again upon encountering the same Ag in the CNS. By preimmunizing rats, a critical number of Ag-specific B and T lymphocytes have already been generated. These cells are available to traffic shortly following CSF infusion of Ag and may even be part of the existing recirculating lymphocytes, competent to cross cerebral endothelial or epithelial boundaries, at the time of Ag infusion into the brain.

In previous studies, we have identified two peripheral sources of Ab-secreting cells, the cervical lymph nodes and the spleen, elicited by Ag infusion into brain parenchyma or CSF (4), similar to other results (23, 24). Kida et al. (25) have also shown that, in addition to cervical lymph nodes, lumbar lymph nodes draining the spinal cord collect ventricular CSF-infused India ink particles, but these authors did not assess Ab responses. Thus, remaining to be defined are the relative roles that lymph nodes and spleen play in induction plus maturation of the peripheral Ab response and in cell trafficking. Before infusion of T-dependent Ag into the brain, removal of cervical lymph nodes blunts the serum Ab response to brain-infused Ag, yet removal of the spleen does not (4, 26). Ag infused into brain reaches cervical nodes and spleen via drainage along the olfactory nerve into the cervical lymph and through arachnoid villi into the blood, respectively. However, concentrations of draining protein Ag in cervical lymph are much greater than in the blood (27); potentially so are the cell populations in nodes vs spleen with which Ag interacts (evidenced by the peripheral Ab response to a T-independent Ag) (26). It is possible that cervical nodes play a more prominent role early in the induction of Ab responses to T-dependent Ags. Following induction, Ag-activated B cells and/or Th cells from the cervical nodes could then traffic to the spleen, reencounter draining Ag, and elicit further changes leading to secretion of Abs by cells in the spleen.

Furthermore, it is not known at what developmental stage the peripheral B cells are able to traffic from secondary lymphoid organs to the brain. Based upon similarities that may exist with T cells (8), it is tempting to speculate that stimulation of peripheral B cells by Ag plus CFA and/or reintroduction of soluble Ag into the brain elicits B lymphoblast transformation, and these lymphoblasts (both B and T) are competent to circulate, randomly seeking Ag in any tissue. The presence of Ag in the CNS leads to retention of these blast cells at the site of deposition, and subsequently, to formation of plasma cells. Others have detected B lymphoblasts in
the efferent lymphatic circulation, and they may be intermediates in the trafficking of B cells from germinal centers of secondary lymphoid organs to other sites in the body where specific Ag is present (17).

Our immunohistochemistry data clearly demonstrate that the presence of plasma cells is dependent upon Ag introduction into the brain. Remarkably, there is no indication of widespread disruption of the BBB at the time when B cells and plasma cells are detected in the brain. But, examination of brain tissue within the vicinity of Ag introduction does reveal a number of changes from normal brains, besides the presence of plasma cells and B cells, viz an enhanced state of reactivity of resident parenchymal cell elements. Some changes are a consequence of cannula implantation, producing a delimited parenchymal wound eliciting a restricted microglial and astrocytic response as part of the healing process. Additionally, locally devitalized tissue would require removal; thus, a population of macrophages would be expected. These wound-healing changes are also observed in cannula only and saline-infused controls and are well advanced toward resolution at the times of observation. The other changes we observe, only in Ag-stimulated brains, demonstrate the presence of an immune reactive region, but in no way did it resemble in severity or extent the inflammatory responses observed in EAE, a well-characterized, T cell-mediated disease. The reactions we observe are far milder and more diffuse. The presence of high levels of circulating anti-OVA Abs and activated lymphocytes to the immunizing Ag made no detectable difference in this cellular response.

Comparing the pericranial areas of saline-infused to Ag-infused brains, a dramatic increase in the area and staining density of IgG (Fig. 4), including anti-OVA Abs detected with FITC-OVA, was observed. The degree of coextensive labeling with anti-IgG and FITC-OVA was more pronounced in OVA-infused preimmunized rats. Spreading of immunospecific activity along the cannula track is attributed to diffusion of infused Ag along the path of least resistance. One possible explanation is that by the time the tissue was examined, local production of Ag-specific Ab (by plasma cells that had differentiated behind the intact BBB) was sufficient to give the area a diffuse IgG positivity. Alternatively, although there is no indication of widespread disruption of the BBB, one cannot totally exclude the possibility of a focal site of increased barrier permeability. Colocalization of Ag-specific B and T cells, plus release of cytokines, could enhance cellular infiltration at the site and local seepage of plasma Ig.

A comparison of paired CSF and serum samples for anti-HSA by IEF and immunoblotting demonstrates an important difference (Figs. 2 and 3). Oligoclonal Ab banding was detectable in the CSF of rats receiving HSA by microinfusion with or without preimmunization. Oligoclonality is indicative of the expansion of certain B cell clones within the CNS and has been observed in a number of diseases that involve the CNS, e.g., multiple sclerosis and neurosyphilis (28, 29). However, estimating that intrathecal synthesis accounts for at least 50% and possibly up to 80% of the anti-OVA in the CSF of rats receiving HSA by microinfusion (Table II, day 5), expansion of B cell clones is more widespread, i.e., polyclonal. Preferential expansion of a few B cell clones, to account for the bands, may be due to several factors, including proximity to a source of stimulation (Ag, cytokines) and affinity for or dosage of infused Ag. It is unknown whether the process leading to intrathecal B cell expansion and differentiation to plasma cells must be initiated by activated Ag-specific T lymphocytes, since HSA and OVA are T-dependent Ags. T cells that have detected their cognate Ag during passage through the brain are retained for a finite period (8, 9) and may attract other elements of the immune response. Since CD4\(^+\) and CD8\(^+\) lymphocytes were readily detectable near the cannula, this is a possibility. Ag-specific B cells finding their way to the site are probably already activated (resting lymphocytes do not cross normal endothelia (30)) and competent to differentiate into plasma cells upon encountering their cognate Ag in the tissue. Although T cells are the most likely candidates for stimulating cell population differentiation, the possibility that B cells obtain sufficient “help” from other cellular elements resident in the parenchyma cannot be excluded.

Issues of B cell traffic to the brain are more significant in light of two recent reports. B cell-deficient mice exposed to encephalitogenic peptide develop EAE but are unable to down-regulate the disease (31). A role for B cells in neuroinvasion is also evident using a mouse model of the prion disease scrape, as B cell-deficient mice fail to develop the brain pathology when prion protein is introduced by the i.p., route but are susceptible when exposed by the intracerebral route (32).

In summary, the results of this study provide evidence to strongly support the conclusion that infusion of a T-dependent protein Ag into a brain with normal barrier permeability can lead to an intrathecal humoral immune response. The sources of lymphocytes mediating this response are secondary lymphoid organs stimulated by the Ag. The primary antigenic stimulus of the lymphoid organs may be derived from the site of CNS immunization (4, 31) or introduced by peripheral injection (this study). An important issue appears to be induction of a pool of migrating, activated lymphocytes capable of entering the normal CNS. Retention of migrating B lymphocytes within the CNS and their subsequent interactions leading to Ab secretion, for example, is clearly dependent upon the presence of Ag within the CNS. This model of inducing an Ab-specific immune response provides a powerful tool for dissecting the critical cellular, cytokine, and costimulatory signals that govern B cell/Ab responses within the brain. In conclusion, we propose that activated B cells, like T cells, are competent to locate their specific Ags at any site in the body—even within the central nervous system.

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This study was written when Dr. Csern was working with stricken with a terminal cancer. We dedicate this paper to her memory.

References


