Inhibition of Experimental Autoimmune Encephalomyelitis by a Tyrosine Kinase Inhibitor

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Inhibition of Experimental Autoimmune Encephalomyelitis by a Tyrosine Kinase Inhibitor

Gabriela Constantin, Carlo Laudanna, Stefan Brocke, and Eugene C. Butcher

Migration of lymphocytes from the blood into the brain is a critical event in the pathogenesis of experimental autoimmune encephalomyelitis. Lymphocyte adhesion to brain endothelium is the first step in lymphocyte entry into the central nervous system, leading subsequently to myelin damage and paralysis. In this paper we show that the tyrosine kinase inhibitor, tyrphostin AG490, prevents binding of freshly isolated mouse lymph node cells and of in vivo activated lymphocytes to endothelium of inflamed brain in Stamper-Woodruff adhesion assays. Moreover, AG490 inhibits adhesion of encephalitogenic T cell lines to purified ICAM-1 and VCAM-1, molecules implicated in T cell recruitment into the central nervous system. In contrast, 2-h treatment of T cell lines with high doses of tyrphostin AG490 have no effect on the viability, intracellular calcium elevation induced by Con A or TCR cross-linking, proliferation, or TNF production by Ag-stimulated T cell lines. Systemic administration of AG490 prevents the accumulation of leukocytes in the brain and the development of experimental autoimmune encephalomyelitis induced by proteolipid protein, peptide 139–151-specific T cell lines in SJL/J mice. Blood leukocytes isolated from mice treated with tyrphostin AG490 are less adhesive on purified very late Ag-4 ligands compared with adhesion of leukocytes from control animals. Our results suggest that inhibition of signaling pathways involved in lymphocyte adhesion may represent a novel therapeutic approach for demyelinating diseases. The Journal of Immunology, 1999, 162: 1144–1149.

Materials and Methods

In vitro binding assays on inflamed brain sections

Normal lymphocytes were freshly isolated from peripheral lymph nodes, while in vivo activated lymphocytes were obtained from draining lymph nodes from SJL mice immunized in the footpads with CFA 10 days earlier (5). Lymphocytes were treated with 100 µM genistein (LC Services, Woburn, MA) for 1 h or with 150 µM tyrphostin (LC Services) for 2 h in RPMI supplemented with 1 mM pyruvate, 1 mM glutamine, 5 x 10^{-5} M 2-ME, and 10% BCS (HyClone, UT) containing 1% iron. Cells were pelleted and resuspended in binding assay medium represented by DMEM without sodium bicarbonate, containing 10 mM HEPES and 5% RPMI supplemented with 1 mM sodium pyruvate, 1 mM glutamine, 5 x 10^{-5} M 2-ME, and 10% BCS (HyClone, UT) containing 1% iron. Cells were pelleted and resuspended in binding assay medium represented by DMEM without sodium bicarbonate, containing 10 mM HEPES and 5% BCS (HyClone, UT), pH 7.2, at a concentration of 5 x 10^7/100 µL. Binding assays were performed for 30 min at 25°C on freshly cut, unfixed, serial frozen sections from SJL mice with clinical EAE (5). The slides, containing two serial sections each (one for nontreated cells and one for cells treated with PTK inhibitor), were gyrated on a platform at 60 rpm. Binding
of control and treated cells was quantitated on the same vessels in serial brain sections. Fifteen to twenty inflamed vessels were counted per slide, and 10–15 slides were used for each experimental condition. We evaluated only the vessels that had at least five adherent cells on control sections, as previously described (5). Analyses were performed in a single blind fashion.

**In vitro binding assay on purified proteins**

Slides were coated overnight at 4°C with purified mouse ICAM-1, VCAM-1, and fibronectin and were blocked for 10 min at 25°C with FBS. T cell lines were Ag stimulated for 3 days, and then a Ficol gradient was performed. Viable T cell lines were treated or not treated with 100 μM tyrphostin AG490 or tyrphostin AG1478 for 2 h and then were added at 60 × 10⁶/25 μl/well and incubated for 20 min at 37°C. Binding assay medium was represented by DMEM without sodium bicarbonate, containing 10 mM HEPES and 5% BCS (HyClone, UT), pH 7.2. Then the slides were washed in PBS and fixed. Computer-assisted enumeration was performed (12). In other experiments, 0.3-ml blood samples were obtained from animals treated with AG490 or DMSO/DMEM 4–6 h after the last drug administration. Blood was collected in an Eppendorf tube containing PBS/heparin and 1% dextran to precipitate RBC; after 25 min, supernatants containing leukocytes were collected and centrifuged for 5 min at 400 × g, and plasma and leukocytes were separated (13). Cells were added at 100 × 10⁶/25 μl/well and incubated for 20 min at 37°C. Slides were washed and fixed, and then computer-assisted enumeration was performed. Back-ground binding was minimal in all experiments and was subtracted.

**Measurement of intracellular Ca²⁺ release**

For TCR cross-linking 5 × 10⁶ T cell lines were Ag stimulated for 3 days, treated for 2 h with 200 μM tyrphostin AG490 at 37°C, pelleted, and then treated for 1 h in ice in PBS with a hamster anti-mouse TCR (PharMingen, San Diego, CA) at 20 μg/ml. After two washings, cells were resuspended in HBBS. After 30–60 s of stirring at 37°C in the cuvette of a Perkin-Elmer LS-50 luminescence spectrometer (Perkin-Elmer, Norwalk, CT), goat anti-hamster F(ab)₂ was added at a final concentration of 25 μg/ml.

**Proliferation assay**

PLP₁₃₉–₁₅₁-specific T cell lines (G1 and G2) were Ag stimulated for 3 days and then treated for 2 h with various concentrations of tyrphostin AG490. Cells were pelleted and seeded at 5 × 10⁶/well in flat-bottom tissue culture 96-well plates for 16 h in fresh medium containing Ag. [³H]thymidine (1 μCi) was added in each well 8 h before the cultures were terminated. Cells were then collected, and samples were counted in a liquid scintillation counter.

**TNF-α measurement**

PLP₁₃₉–₁₅₁-specific T cell lines (G1 and G2) were Ag stimulated for 3 days and then treated for 2 h with various concentrations of tyrphostin AG490. Cells were pelleted and resuspended in fresh medium containing Ag at a concentration of 5 × 10⁶ cells/ml for 24 h. Supernatants were harvested, and TNF-α was measured using an immunoassay kit for mouse TNF-α (BioSource International, Camarillo, CA).

**Transfer and evaluation of EAE**

SJL/J females, 6–8 wk old, were purchased from The Jackson Laboratory (Bar Harbor, ME). The production, characterization, and maintenance of G1 and G2 PLP₁₃₉–₁₅₁-specific T cell lines were previously described (14, 15). In brief, SJL/J mice were immunized with 250 μg of peptide PLP₁₃₉–₁₅₁ in CFA. Ten days later, draining lymph nodes were removed and stimulated with 30 μg/ml peptide for 4 days. T cell lines were obtained by stimulation of these cultures every 14 days with irradiated syngenic tissue culture slides for 5 days. T cell lines were cultured with protein tyrosine kinase inhibitors. Cell treatment and binding assays were performed as described in Materials and Methods. Numbers represent the percentage of binding relative to the control with nontreated cells ± SEM. For Tyr.AG490, the same experimental protocol was used as above. The inhibitory effect of AG490 was dose dependent. For the bottom two entries, the production, characterization, and maintenance of PLP₁₃₉–₁₅₁-specific T cell lines was as previously described in Refs 14 and 15. Binding assays were assessed on 18-well glass slides coated with purified mouse ICAM-1, VCAM-1, and fibronectin. Values are the mean counts of bound T cells per 0.2 mm² in three experiments (three wells/condition) ± SDs.

**Table I. Protein tyrosine kinases inhibitors block lymphocyte binding in vitro**

<table>
<thead>
<tr>
<th>PTK Inhibitors</th>
<th>Normal lymphocytes</th>
<th>Activated lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistein</td>
<td>57 ± 5</td>
<td>49 ± 8</td>
</tr>
<tr>
<td>Tyr.AG490</td>
<td>13 ± 3</td>
<td>10 ± 5</td>
</tr>
<tr>
<td>Tyr.AG555</td>
<td>21 ± 9</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>Tyr.AG1478</td>
<td>105 ± 10</td>
<td>105 ± 5</td>
</tr>
<tr>
<td>Tyr.AG82</td>
<td>79 ± 5</td>
<td>85 ± 2</td>
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<tr>
<td>Tyr.AG213</td>
<td>101 ± 4</td>
<td>90 ± 11</td>
</tr>
<tr>
<td>Tyr.AG18</td>
<td>60 ± 10</td>
<td>65 ± 10</td>
</tr>
<tr>
<td>Tyr.AG183</td>
<td>73 ± 1</td>
<td>80 ± 2</td>
</tr>
<tr>
<td>Tyr.AG126</td>
<td>62 ± 8</td>
<td>60 ± 4</td>
</tr>
<tr>
<td>Tyr.AG879</td>
<td>90 ± 6</td>
<td>88 ± 11</td>
</tr>
</tbody>
</table>

For genistein and tyrphostins, binding assays were performed on inflamed brain serial sections using normal and in vivo-activated lymphocytes previously treated with protein tyrosine kinase inhibitors. Cell treatment and binding assays were performed as described in Materials and Methods. Numbers represent the percentage of binding relative to the control with nontreated cells ± SEM. For Tyr.AG490, the same experimental protocol was used as above. The inhibitory effect of AG490 was dose dependent. For the bottom two entries, the production, characterization, and maintenance of PLP₁₃₉–₁₅₁-specific T cell lines was as previously described in Refs 14 and 15. Binding assays were assessed on 18-well glass slides coated with purified mouse ICAM-1, VCAM-1, and fibronectin. Values are the mean counts of bound T cells per 0.2 mm² in three experiments (three wells/condition) ± SDs.

**Results**

The association of tyrosine phosphorylation events with integrin-mediated adhesive interactions suggested that PTK inhibitors might modulate lymphocyte adhesion to brain endothelium. We performed in vitro binding assays on serial unfixed frozen sections of EAE brains, using a modification (5, 16) of the original technique described by Stamper and Woodruff (17). Freshly isolated resting lymph node cells and in vivo activated lymphocytes bound selectively to vessels from inflamed brain (3), but not to any vessels in sections of control brains. Various PTK inhibitors were examined for inhibitory activity in the in vitro assay (Table I). We first tested genistein, a widely used competitive inhibitor of the ATP binding site (10); genistein inhibited 43 and 50% of the attachment of freshly isolated or in vivo activated lymphocytes to inflamed brain endothelium. We then analyzed a panel of more selective low m.w. substrate-mimicking compounds, the tyrphostins. Many of these inhibitors have distinctive inhibitory ac-

<table>
<thead>
<tr>
<th>Binding to purified proteins</th>
<th>Binding to inflamed vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr.AG490</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>Tyr.AG1478</td>
<td>98 ± 3</td>
</tr>
</tbody>
</table>

For genistein and tyrphostins, binding assays were performed on inflamed brain serial sections using normal and in vivo-activated lymphocytes previously treated with protein tyrosine kinase inhibitors. Cell treatment and binding assays were performed as described in Materials and Methods. Numbers represent the percentage of binding relative to the control with nontreated cells ± SEM. For Tyr.AG490, the same experimental protocol was used as above. The inhibitory effect of AG490 was dose dependent. For the bottom two entries, the production, characterization, and maintenance of PLP₁₃₉–₁₅₁-specific T cell lines was as previously described in Refs 14 and 15. Binding assays were assessed on 18-well glass slides coated with purified mouse ICAM-1, VCAM-1, and fibronectin. Values are the mean counts of bound T cells per 0.2 mm² in three experiments (three wells/condition) ± SDs.

Statistical evaluation

Comparisons between groups were made using Student’s t test.
suggesting that AG490 has a specific effect on lymphocyte adhesion. We then evaluated the effect of AG490 on adhesion of encephalitogenic PLP139–151-specific T cell lines to purified ICAM-1, a ligand for αLβ2, and to VCAM-1 and fibronectin, ligands for α4β1 (Table I). AG490 inhibited adhesion to VCAM-1 and fibronectin by 93%, and inhibited binding to ICAM-1 by 88%. Thus, AG490 inhibits adhesion events that are thought to be critical to the development of EAE. FACS analyses revealed no change in the expression of LFA-1, VLA-4, CD44, ICAM-1, CD45, CD4, or CD3 on AG490-treated T cell lines (not shown). As an additional control, we assessed the effect of AG490 on intracellular Ca2+ elevation induced by Con A or stimulated through CD3 cross-linking, a signaling event dependent on PTK of the Src family but not on JAK-2 (23). As shown in Fig. 2A, AG490 had no effect on these responses, ruling out nonspecific toxicity.

We next analyzed the effect of AG490 on the proliferation of G1 and G2 T cell lines specific for PLP139–151. AG490 treatment had no effect on the incorporation of [3H]thymidine by our Ag-stimulated T cell lines after 2 h of drug treatment (Fig. 2B), a finding in agreement with recent studies demonstrating unaltered proliferation of AG490-treated mitogen-stimulated T and B cells (19). However, when the proliferation assays were performed in the presence of the drug for 24 h, starting at 20 μM AG490 there was a partial (38%) inhibition of proliferation, while at high doses (>100 μM) the proliferation was massively blocked, and substantial toxicity was observed, with cell death (up to 50%; data not shown). The doses of AG490 we used in vivo (see the results

**FIGURE 1.** In vitro binding of lymphocytes to inflamed vessels in EAE mouse brain. Nontreated lymph node lymphocytes bound to inflamed vessels, to the vascular lumen (A), as previously described (3). In the same experiment, lymphocytes pretreated with 150 μM tyrphostin AG490 were incubated on serial sections of inflamed brain. AG490 inhibited lymphocyte binding to the inflamed venule (B). In serial inflamed brain sections, no significant difference was observed between binding of nontreated cells (C) and that of tyrphostin 1478-treated lymphocytes (D). See also Table I.

![Image](http://www.jimmunol.org/)

**FIGURE 2.** Effects of tyrphostin AG490 on calcium release, proliferation, and TNF-α production. A, Increase in cytosolic free calcium in PLP139–151-specific T cell lines after treatment with Con A or TCR cross-linking. Assays were conducted as described previously (26). Fura-2/AM-loaded T cell lines stimulated with 100 μM Con A (arrow) showed no difference in the increase in intracellular calcium after treatment with 200 μM tyrphostin AG490 for 2 h. AG490 also had no effect on Ca2+ mobilization by TCR cross-linking (arrow). B, The effect of AG 490 on Ag-induced proliferation was determined as described in Materials and Methods. C, TNF-α release was measured in supernatants from Ag-stimulated T cell lines treated with AG490 as described in Materials and Methods.
below) were 1.5 (intradermally (i.d.)) and 3.5 mg/day (i.d. plus i.p.), and from the literature (19), this treatment should lead to transient peak drug concentrations in the blood of 5–10 μM. At doses of 5–10 μM we found no significant effect of AG490 on induced T cell proliferation in culture for up to 24 h, even when AG490 was continuously present (data not shown).

The ability of T cell lines specific for myelin Ags to transfer EAE was positively correlated with the amount of proinflammatory cytokines such as TNF-α and lymphotoxin-α (24). We found that TNF-α production by our Ag-stimulated G1 and G2 PLP139–151-specific T cell lines was unaffected by AG490 treatment (Fig. 2C).

Finally, the viability of G1 and G2 T cell lines specific for PLP139–151 was assessed using the nuclear fluorescent dye YO-Pro-1 (25). After 3 days of Ag stimulation, T cells were washed and incubated further with or without 200 μM AG490 for 2 h. After washing and culture for an additional 3 or 6 days, AG490-treated cultures contained 45 and 50%, respectively, more viable T cells, as assessed by flow cytometry of CD3-positive cells (data not shown). This observation is consistent with the reported ability of AG490 to increase the viability of germinal center B lymphocytes in vitro (26). Together, these results excluded nonspecific toxicity.

As tyrphostin AG490 blocked lymphocyte adhesive interactions implicated in EAE pathogenesis, we next determined its effects on disease (Table II). Encephalitogenic T cell lines specific for PLP139–151 were treated with tyrphostin AG490 and injected i.v. into SJL mice. Because tyrphostins are reversible inhibitors, mice also received 50 μg of AG490 to the time of T cell transfer and 1.5 mg/day i.d. (Table II, Expt. 1). AG490 completely prevented the development of paralysis in all treated animals. Immunohistochemically, brains from untreated animals revealed extensive leukocyte infiltration (Fig. 3A), whereas infiltration was not detected in brains from animals treated with AG490 (Fig. 3B). In other experiments, untreated T cell lines were injected into mice treated daily with AG490 i.d. or/and i.p. Under these experimental conditions, the protective effect of tyrphostin was dose dependent. When 1.5 mg of tyrphostin was administered i.d. daily beginning on day 0, all mice developed disease but the day of onset was delayed (p < 0.001), and disease severity was reduced (p < 0.005; Table II, Expt. 2). When mice received 1.5 mg drug i.d. and 2 mg i.p. (3.5 mg daily in total) beginning at day 0, control animals received either DMSO/DMEM i.d. or no treatment. Animals received the treatment for 25 days posttransfer. Only diseased animals were considered to calculate the day of onset and the clinical score. Animal care was in accordance with our institutional guidelines. Results are expressed as the mean ± SD. Differences in clinical score between DMSO/DMEM-treated animals and AG490-treated animals were evaluated for statistical significance: p < 0.005 in Expt. 2; p < 0.001 in Expt. 3.

Table II. In vivo administration of tyrphostin AG490 prevents paralysis

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Agent</th>
<th>Pretreatment of T Cell Lines</th>
<th>Treatment of Recipients</th>
<th>Incidence of the Disease</th>
<th>Day of Onset</th>
<th>Maximal Clinical Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>/</td>
<td>−</td>
<td>−</td>
<td>6/6</td>
<td>11.1 ± 1</td>
<td>2.5 ± 0.5</td>
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<tr>
<td></td>
<td></td>
<td>DMSO/DMEM</td>
<td>+</td>
<td>7/7</td>
<td>10.7 ± 1.5</td>
<td>2.4 ± 0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AG490</td>
<td>+</td>
<td>0/7</td>
<td>−</td>
<td>0</td>
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<td></td>
<td>(1.5 mg/day *)</td>
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<tr>
<td>2</td>
<td>/</td>
<td>−</td>
<td>−</td>
<td>5/5</td>
<td>5.6 ± 0.6</td>
<td>3 ± 1</td>
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<tr>
<td></td>
<td></td>
<td>DMSO/DMEM</td>
<td>−</td>
<td>7/7</td>
<td>5.3 ± 0.9</td>
<td>3.1 ± 0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AG490</td>
<td>+</td>
<td>7/7</td>
<td>10.1 ± 1.7</td>
<td>1.8 ± 0.6</td>
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<tr>
<td></td>
<td></td>
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<td></td>
<td>(1.5 mg/day)</td>
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<tr>
<td>3</td>
<td>/</td>
<td>−</td>
<td>−</td>
<td>5/5</td>
<td>6.6 ± 0.9</td>
<td>3.2 ± 0.3</td>
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<tr>
<td></td>
<td></td>
<td>DMSO/DMEM</td>
<td>−</td>
<td>6/6</td>
<td>6.3 ± 0.5</td>
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<tr>
<td></td>
<td></td>
<td>AG490</td>
<td>−</td>
<td>3/6</td>
<td>11 ± 2.6</td>
<td>1.2 ± 0.6</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(3.5 mg/day)</td>
<td></td>
</tr>
</tbody>
</table>

Production, characterization, and maintenance of G1 and G2 PLP139–151-specific T cell lines was as previously described (14, 15). Expt. 1: G1 T cells were Ag stimulated for 3 days and treated for 2 h with 150 μM tyrphostin AG490 or DMSO. Cells (5 × 105) were i.v. injected into SJL mice. Mice injected with AG490-treated cells also received 50 μg AG490 at the time of transfer (*) and 1.5 mg/day i.d. in DMSO/DMEM. Control animals received either DMSO/DMEM i.d. or no treatment. Animals received the treatment for 25 days posttransfer. Expt. 2: G2 T cell lines (3 × 105) were stimulated with PLP139–151 for 3 days and injected i.v. without receiving any drug treatment. Mice were treated daily with 1.5 mg AG490 i.d. in DMSO/DMEM, beginning at day 0. Control animals received either DMSO/DMEM i.d. or no treatment. Animals were treated for 25 days posttransfer. Expt. 3: Mice receiving G2 cells were treated daily with 1.5 mg AG490 i.d. and 2 mg i.p. (3.5 mg daily in total) beginning at day 0. Control animals received either DMSO/DMEM i.d. and i.p. or no treatment. Animals received the treatment for 25 days posttransfer. Only diseased animals were considered to calculate the day of onset and the clinical score. Animal care was in accordance with our institutional guidelines. Results are expressed as the mean ± SD. Differences in clinical score between DMSO/DMEM-treated animals and AG490-treated animals were evaluated for statistical significance: p < 0.005 in Expt. 2; p < 0.001 in Expt. 3.

FIGURE 3. In vivo administration of tyrphostin AG490 prevents accumulation of leukocytes in the CNS. Brains were removed from several diseased animals and from AG490-treated animals with no clinical signs 20 days after transfer. The micrographs show hematoxylin-eosin-stained sections of comparable regions of the brain stem from animals from Expt. 1 (Table II). There was extensive inflammatory infiltration in brains from diseased animals (A; arrows), whereas no infiltrates could be detected in parenchyma of CNS from mice treated with AG490 (B).
Previous studies have demonstrated that mAb inhibitors of adhesion molecules, including integrin α4β1, prevent the development of EAE in the murine model (3, 4). Here we present evidence that inhibition of signal transduction pathways involved in lymphocyte adhesion may also be useful in treating inflammatory diseases of the central nervous system. As lymphocyte entry into the brain is thought to represent a critical moment in the pathogenesis of EAE, we first conducted in vitro studies to find second messenger inhibitors able to interfere with lymphocyte adhesion to brain endothelium or to ligands considered important in this phenomenon. The results suggested that PTKs are involved in lymphocyte adhesion to brain endothelium. Among different tyrphostins and other PTK inhibitors tested, tyrphostin AG490, a selective inhibitor of JAK-2 kinase (19), was the most effective inhibitor of lymphocyte binding to inflamed vessels. FACS analyses revealed no change in the expression of adhesion molecules on AG490-treated T cell lines, showing that the effect of AG490 on lymphocyte adhesion is not due to altered expression of adhesion molecules per se, but to an inhibition of signaling pathways leading to integrin-dependent lymphocyte adhesion.

We then asked whether this inhibition might have clinical relevance in an EAE model. AG490 was able to prevent the transfer of EAE by encephalitogenic T cell lines, and this inhibitory effect of AG490 on the development of EAE was associated with a decreased adhesion of blood leukocytes to VCAM-1 and fibronectin, both ligands of the integrin VLA-4. In contrast, in vitro the same AG490 treatment applied to T cells before injection into the mice had no effect on the viability, intracellular calcium elevation in-
AG490 is a potent and reportedly selective inhibitor of JAK-2 kinase, having no effect on the kinase activity of other PTKs, such as Src, Lck, Lyn, Btk, and Syk (19). Moreover, JAK-2 is a PTK involved in signaling by cytokines such as IL-3, granulocyte-macrophage CSF, and TNF, that are themselves able to activate integrin function and promote cell adhesion (28–30). Thus, JAK-2-dependent pathways may be involved in the adhesion of T cells to integrin ligands expressed on brain endothelium and may represent the AG490 target whose inhibition blocks EAE.

In conclusion, although we can never formally exclude the possibility that the in vivo effects of AG490 on disease can be mediated through inhibition of adhesion and additional mechanisms implicated in other aspects of the pathobiology of EAE, our results suggest that therapies designed to interfere with signal transduction mechanisms involved in integrin-dependent lymphocyte adhesion may be useful in treating autoimmune demyelinating diseases. Antibodies directed against α4 and β2 integrins have been reported to modulate other autoimmune and inflammatory diseases, as well, suggesting that this novel approach may have broader application to immune system pathology.

References