Immunosuppressant drugs such as cyclosporin have allowed widespread organ transplantation, but their utility remains limited by toxicities, and they are ineffective in chronic management of autoimmune diseases such as multiple sclerosis. In contrast, the immune modulating drug FTY720 is efficacious in a variety of transplant and autoimmune models without inducing a generalized immunosuppressed state and is effective in human kidney transplantation. FTY720 elicits a lymphopenia resulting from a reversible redistribution of lymphocytes from circulation to secondary lymphoid tissues by unknown mechanisms. Using FTY720 and several analogs, we show now that FTY720 is phosphorylated by sphingosine kinase; the phosphorylated compound is a potent agonist at four sphingosine 1-phosphate receptors and represents the therapeutic principle in a rodent model of multiple sclerosis. Our results suggest that FTY720, after phosphorylation, acts through sphingosine 1-phosphate signaling pathways to modulate chemotactic responses and lymphocyte trafficking.

FTY720 is derived from ISP-1 (myriocin), a fungal metabolite that is an eternal youth nostrum in traditional Chinese herbal medicine (1). The compound (2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol) is a novel, high potency immune modulating agent that is remarkably effective in a variety of autoimmune and transplant models including islet transplantation (2) and has recently been proven to be effective in renal transplantation in man (3). Unlike the currently used immunosuppressive agents (e.g. the calcineurin inhibitors cyclosporin and tacrolimus), FTY720 does not inhibit T cell activation and proliferation and in rodent models does not impair immunity to systemic viral infection (4). If confirmed in man, the latter property provides a striking advantage over current immunosuppressive therapies. FTY720 apparently sequesters lymphocytes from circulation to secondary lymph tissue compartments (5) with concomitant reduction of specific effector T cells recirculating from the lymph nodes to inflamed peripheral tissues (4) and graft sites (6). FTY720 does not act via the lymphocyte-homing chemokine receptor CCR-7 because FTY720 is active both in CCR-7-deficient mice and plt (paucity of lymph node T cells) mice, which lack CCR-7 ligands (CCL-19 and CCL-21) (7).

FTY720-induced lymphocyte homing is sensitive to suppression by pertussis toxin (6–8), which suggests that the molecular target of the drug is a G protein-coupled receptor (GPCR) interacting with heterotrimERIC G proteins of the α1o type. The affected GPCR(s) is on the lymphocyte since fluorescently labeled lymphocytes treated with pertussis toxin ex vivo and transferred to mice are not depleted by FTY720 in vivo (8). The structural similarity of FTY720 and sphingosine has prompted speculation that the drug might act via the sphingosine 1-phosphate (SIP) receptor SIP1 (formerly Edg-6)2 that is known to be expressed by lymphocytes (9).

SIP is a pleiotropic lysosphospholipid mediator; the prominent cellular responses to applied SIP are transient calcium mobilization, inhibition of adenyl cyclase, escape from apoptosis (10), increased cell migration (11, 12), and mitogenesis (13). The physiologic role of SIP remains undefined although cell culture experiments and the phenotype of a mouse with the SIP1 receptor gene ablated suggest a role for SIP in vascular maturation (14, 15). Responses to SIP are mediated through a set of five cell surface GPCRs (SIP1–5), and the various effects of SIP have been attributed to interactions with one or more of these receptors (16). SIP is formed by the action of sphingosine kinase on sphingosine (17). The activity of this enzyme is increased in response to external stimuli (18, 19), and enforced expression of sphingosine kinase increases both cell proliferation and survival (20). Sphingosine is converted rapidly to SIP when added to cells (21), while the route of SIP degradation to sphingosine might proceed via an ectophosphatase (22). To learn whether FTY720 might participate in the sphingosine-SIP signaling cascade, we performed the studies described herein.
FTY720 and Sphingosine 1-Phosphate Signaling

EXPERIMENTAL PROCEDURES

Sphingosine Kinase Assay—The assays were performed as described previously (23) using mouse recombinant sphingosine kinase 1a expressed in Escherichia coli. The reaction buffer contained 50 mM Hepes, pH 7.4, 15 mM MgCl₂, 10% glycerol, and 0.05% Triton X-100. Substrates (sphingosine, FTY720, or AAL) were incubated at various concentrations with 10 mM sphingosine kinase, 10 μM ATP/0.5 μl of [γ-35S]ATP (3000 Ci/mmol; Amersham Biosciences) at 30°C for 1 h. Lipids were extracted with 2 volumes of CHCl₃/methanol (1:2), the organic extraction product was dried, and the pellet was redissolved. The lipids were separated on a thin layer chromatography plate using a 1-butanol/acetic acid/water (6:2:2) solvent system after which the plate was exposed to x-ray film to detect phosphorus-32-labeled lipids.

Measurement of Circulating Lymphocytes—FTY720 and AAL were dissolved in water and administered by gavage to Lewis rats at various doses. FTY720-P and AFD were dissolved in water:Me₃SO (5:1, v/v) and injected intraperitoneally into C57BL/6 mice (1 mg/kg). Blood was collected from the tail vein of mice or the sublingual vein of rats 8 h after drug administration and subjected to hematology using an automated Techicon H1-E analyzer (Bayer Diagnostics, Unterschleissheim, Germany).

Apopotosis Assay—Human CD4⁺ T cells were negatively selected from Ficoll-isolated peripheral blood mononuclear cells by magnetic cell sorting according to standard procedures using anti-CD8-, anti-CD20-, and anti-CD14-coated Miltenyi Biotec (Gladbach, Germany) magnetic beads. CD4⁺ T cells (10⁶/ml) were incubated with increasing concentrations of compounds for 4 h. Cells were stained for expression of phosphatidylserine in the outer leaflet of the membranes using the Annexin-V-FLUOS staining kit (Roche Molecular Biochemicals), and positive populations were detected by fluorescence-activated cell sorting.

EXPERIMENTAL Autoimmune Encephalomyelitis (EAE) Model—Wistar rats were immunized with an emulsion of bovine spinal cord in complete Freund’s adjuvant as described previously (24). Two-week oral treatment with FTY720 (aqueous solution) or the enantiomers (dissolved in water:Me₃SO, 10:1, v/v) was started on day 0 using a dose of 0.3 mg/kg/day. Positive controls received vehicle alone. Animals (10 per group) were monitored daily and graded according to disease symptoms.

RESULTS

We used a set of FTY720-like compounds to determine sphingosine kinase and S1P receptor activity and correlate these with assays of lymphocyte function. In addition to FTY720, we tested both enantiomers (AAL) of an analog described by Kiuchi et al. (26) wherein a hydroxymethylene substituent of FTY720 was replaced by a methyl group (Fig. 1). These enantiomers had very different activities; the IC₅₀ values for decreasing circulating T lymphocytes in rats were reported to be 0.009 and >1 mg/kg for the R- and S-enantiomers, respectively, while the ID₅₀ value for FTY720 in the same system was 0.024 mg/kg (26).

We first asked whether these compounds were substrates for sphingosine kinase. Recombinant mouse sphingosine kinase 1a catalyzed the phosphorylation of FTY720 and (R)-AAL but not (S)-AAL (Fig. 2 A and B). Moreover lymphoid tissue including Peyer’s patches, spleen, and lymph nodes effectively phosphorylated FTY720, while heart, liver, and kidney contained little of the phosphorylated drug (Fig. 2C). This pattern of active tissues best matches the RNA localization of sphingosine kinase type 1 (27). The concept that phosphorylated FTY720 might be the active principle is intriguing and suggests that an alcohol/phosphate cycling of FTY720/FTY720-P takes place in vivo as occurs with sphingosine/S1P. Indeed FTY720 was converted extensively to FTY720-P in vivo, resulting in up to 4-fold higher blood levels of FTY720-P compared with parent FTY720 (Table II). To learn whether FTY720-P is dephosphorylated in vivo, we administered single doses of FTY720-P to mice and assayed blood levels of FTY720 after 24 h. FTY720 could be detected after the lowest dose (0.1 mg/kg) of FTY720-P and increased in a dose-dependent fashion (Table I). We next determined whether synthetic phosphate derivatives, namely FTY720-P, (R)-AFD, and (S)-AFD, which resemble S1P (Fig. 1), were agonists at S1P receptors. To interrogate the individual S1P receptors, we used a membrane-based [γ-35S]GTPγS binding assay that allows direct comparison of the rank order potencies (pEC₅₀) and relative efficacies (Eₘax) of agonist ligands at isolated receptors (24). All of our compounds were agonists at the S1P₁ receptor, although FTY720-P and (R)-AFD were far more potent than their non-phosphorylated congeners (Fig. 3). Both FTY720-P and (R)-AFD were high potency agonists also at the S1P₃, S1Pγ, and S1P₄ receptors (Table II), but the corresponding alcohols (FTY720 and AAL) were not efficacious at these three receptors (data not shown). Although FTY720-P behaved as a partial agonist in the [γ-35S]GTPγS binding assay (Table II), this compound was a full agonist in whole cell assays of inhibition of cAMP accumulation where there exists more amplification of signal (data not shown). None of our compounds were active at the S1P₂ receptor in our assays at concentrations up to 10 μM. The receptor activation data are consistent with ligand binding measurements, which demonstrate a high affinity interaction between FTY720-P and (R)-AFD but not (S)-AFD and the S1P receptors (data not shown). Finally the compounds were not active at the three receptors for a structurally related lysosphospholipid mediator, lysosphatidic acid (data not shown).

To learn whether this pattern of activity is recapitulated in vivo, we determined the potency of our compounds in reducing numbers of circulating T lymphocytes. FTY720 and (R)-AAL...
potently reduced circulating T lymphocyte levels in rats in a dose-dependent manner, whereas (S)-AAL and sphingosine were completely inactive at doses up to 1 mg/kg (Fig. 4A). We obtained the analogous result with the respective phosphorylated compounds; a 1 mg/kg bolus injection of FTY720-P or (R)-AFD reduced circulating T cells by about 70%, whereas (S)-AFD and S1P were inactive with this dosing regimen (Fig. 4B). The lymphopenic activity of FTY720 and (R)-AAL in vivo (Fig. 4 and Ref. 25) thus can be explained by their metabolism to the phosphorylated forms (FTY720-P and (R)-AFD), which are potent agonists at multiple S1P receptors. The lack of efficacy of (S)-AAL in decreasing circulating lymphocytes cannot be credited only to its failure as a substrate for sphingosine kinase because even when phosphorylated (synthetically) to form (S)-AFD, it lacks affinity for S1P receptors (Table II).

FTY720 evokes apoptosis in lymphocytes at micromolar concentrations, prompting the idea that the drug acts by killing lymphocytes (28). We consider this mechanism highly unlikely in view of the low nanomolar levels ($C_{\text{max}} < 50 \, \text{nM}$) of FTY720 realized in the blood of rats treated with the high dose of 1 mg/kg (29). Nevertheless we determined whether the apoptotic potential of our compounds correlated with activities in vitro or in vivo. We observed apoptotic responses only in T cells treated with micromolar concentrations of non-phosphorylated compounds (Table III). This pattern is reminiscent of reports of the activity of sphingosine and S1P where sphingosine is associated with apoptosis and S1P is associated with protection from apoptosis (10, 30). The apoptotic responses elicited by the non-phosphorylated compounds were neither stereoselective nor inhibited.

### TABLE I

| Compound administered | Dose | Compound detected$^a$ | Compound detected$^b$ |
|------------------------|------|-----------------------|-----------------------|
|                        | mg/kg | ng/ml                 | FTY720 | FTY720-P |
| FTY720                 | 7.5   | 85 133 73            | 24 179 383 437 109 |
| FTY720                 | 0.1   | ND ND 1.15           | ND ND ND ND ND ND |
| FTY720                 | 0.3   | ND ND 8.90           | ND ND ND ND ND ND |
| FTY720                 | 0.1   | ND ND 0.15           | ND ND ND ND ND ND |
| FTY720                 | 0.3   | ND ND 1.06           | ND ND ND ND ND ND |
| FTY720                 | 1.0   | ND ND 4.91           | ND ND ND ND ND ND |

$^a$ Compound was detected by HPLC separation after extraction from blood pools from three animals.

$^b$ FTY720 administered to Wistar rats (oral).

$^c$ FTY720 or FTY720-P administered to C3H mice (intraperitoneal).

$^d$ ND, not determined.
by prior pertussis toxin treatment (data not shown); both properties contrast the behavior of these compounds in mice or rats regarding the depletion of circulating lymphocytes (7, 26).

The unique mechanism that apparently underlies the immune modulating effects of FTY720, i.e. increased homing of T cells toward the lymphatic system and away from inflammatory tissues (5), provides an opportunity for therapy of autoimmune disorders that does not exist with current immunosuppressive agents. Therefore, we tested FTY720 and both enantiomers of AAL in EAE, which is a primary model of autoimmune disorders that does not exist with current immune modulating effects of FTY720, presumably proceeding either through the non-selective lipid phosphate phosphohydrolases (22) or the S1P phosphatase (30), might be different with resultant differences in accumulation. Alternately FTY720 and (R)-AAL might be more effective substrates of the sphingosine kinase in vivo.

Our discovery that FTY720 can be phosphorylated to yield a potent S1P mimic with substantial implications for S1P biology. This pleiotropic lipid mediator is most often characterized as promoting angiogenesis, cell proliferation, and escape from lymphoid tissue compartments. We have now documented that FTY720 and an analog (R)-AAL, after phosphorylation to FTY720-P and (R)-AFD, are high affinity agonists at four (of five) S1P receptors. The correlation of substrate activity (Fig. 2), agonism at S1P receptors (Fig. 3 and Table II), induction of lymphopenia (Fig. 4), and activity in the EAE model (Fig. 5) suggests strongly that FTY720 and an analog (i.e. (R)-AAL) function ultimately as S1P mimetics that increase the lymphocyte homing response.

The failure of sphingosine and S1P to evoke lymphopenia when administered to rats (Fig. 4B) might relate to an approximately 1 log order higher potency of FTY720-P and (R)-AFD compared with S1P at the S1P1 and S1P2 receptors (Table I) but could also be due to a different metabolic rate for the naturally occurring compound. For example, the rate of dephosphorylation of S1P, FTY720-P, and (R)-AFD, presumably proceeding either through the non-selective lipid phosphate phosphohydrolases (22) or the S1P phosphatase (30), might be different with resultant differences in accumulation. Alternately FTY720 and (R)-AAL might be more effective substrates of the sphingosine kinase in vivo.

Our discovery that FTY720 can be phosphorylated to yield a potent S1P mimic with substantial implications for S1P biology. This pleiotropic lipid mediator is most often characterized as promoting angiogenesis, cell proliferation, and escape from apoptosis. To this list must now be added immune system modulation via changes in lymphocyte trafficking. In addition to increasing knowledge of lysophospholipid medicinal chemistry, our results reinforce the notion that sphingosine participates in a cycle of phosphorylation/dephosphorylation that governs the levels of the alcohol (sphingosine) and phosphate (S1P) forms and thus establishes the S1P tone of tissues. The ability of FTY720 to participate in this cycle to establish an exaggerated
SIP tone probably underlies the high potency and efficiency of this drug. The existence of this cycle and its effect on lymphocyte trafficking might confound development of drugs that are sphingosine kinase inhibitors or SIP receptor antagonists.

FTY720-P and its active analog, (R)AFD, are potent agonists at four SIP receptors, three of which (SIP₁, SIP₄, and SIP₅) are expressed by lymphocytes. Although pertussis toxin treatment of lymphocytes ex vivo demonstrates that a G protein signaling pathway in the lymphocyte is essential for the FTY720-promoted homing response (7, 8), SIP receptors on endothelial cells (including SIP₁ and SIP₄) might participate in the process as well. Thus only the SIP₄ receptor, at which FTY720-P and (R)-AFD are inactive, is eliminated from contention. Likewise we cannot know from present data what sphingosine kinase isoform or what phosphatase isoform is relevant to the metabolism of FTY720. Additional chemical entities and genetically modified mice lacking one or more SIP receptor genes or sphingosine kinase genes are needed to define the FTY720 target(s) more precisely.

An interesting finding reported recently by Hla and colleagues (21) is that sphingosine kinase 1a can be released from cultured cells. Although their experimental system was necessarily artificial and its prediction requires confirmation, taken at face value it suggests the intriguing notion that the phosphorylation of sphingosine might be extracellular. Since the lipid phosphate phosphohydrolases are clearly ectophosphatases (22), the entire cycle might proceed in the extracellular compartment. Such a system would provide a route whereby lysosphospholipid receptors could be accessed using orally available lipid alcohol compounds as exemplified by FTY720.

FTY720 is the first in a class of new immune system modulators that may allow both better management of allograft recipients and more effective treatment of patients with autoimmune disorders, which is a substantially unmet medical need. The drug is apparently less toxic than existing regimens, particularly those used in the treatment of transplantation. While this paper was in review, a report (36) was published describing the metabolism of FTY720 in rodents and the agonist activity of FTY720-P at SIP receptors.

**REFERENCES**

1. Fujita, T., Inoue, K., Yamamoto, S., Ikumoto, T., Sasaki, S., Toyama, R., Chiba, K., Hoshino, Y., and Okumoto, T. (1994) J. Antibiot. 47, 208–215
2. Brinkmann, V., Pinchewer, D. D., Feng, L., and Chen, S. (2001) Transplantation 72, 764–769
3. Tedesco, T., Kahan, B., Mourad, G., Vanrenterghem, Y., Grinyo, J., Weinar, W., Pellet, P., Chodoff, L., and Sablinski, T. (2001) Am. J. Transplant. 1, 1857–1881
4. Pinschewer, D. D., Ochsneben, A. F., Odermatt, B., Brinkmann, V., Hengartner, H., and Zinkernagel, R. M. (2000) J. Immunol. 164, 5761–5770
5. Chiba, K., Yanagawa, Y., Yasubuchi, Y., Katoaka, H., Kagawuchi, T., Ohtsuki, M., and Hoshino, Y. (1998) J. Immunol. 160, 5037–5044
6. Yanagawa, Y., Sugahara, K., Katoaka, H., Kagawuchi, T., Masubuchi, Y., and Chiba, K. (1998) J. Immunol. 160, 5493–5499
7. Brinkmann, V., Chiba, K., and Peng, L. (2000) Trends Pharmacol. Sci. 21, 49–52
8. Graier, M. H., Bernhardt, G., and Lipp, M. (1998) Genomics 53, 164–169
9. Cuvillier, O., Pirianov, G., Kleusser, B., Vanek, P. O., Cuno, O. A., Garkind, J. S., and Spiegel, S. (1996) Nature 381, 809–813
10. Palik, J. H., Chae, S.-S., Lee, M.-J., Thangada, S., and Hla, T. (2001) J. Biol. Chem. 276, 11830–11837
11. Hla, T., Lee, M. J., Ancellin, N., Liu, C. H., Kluk, M., Heuk, M., Milstein, S., and Spiegel, S. (2001) Nature 410, 1800–1803
12. Zhang, H., Desai, N. N., Olivera, A., Seki, T., Brooker, G., and Spiegel, S. (1991) J. Cell Biol. 114, 155–167
13. Lee, J.-M., Chang, S., Claffey, K. P., Ancellin, N., Liu, C. H., Kluk, M., Volpi, M., Shaba’i, R. I., and Hla, T. (1999) Cell 99, 301–312
14. Liu, Y., Wada, R., Yamashita, T., Shi, Y., Deng, C. X., Hla, T., and Hla, T. (2000) J. Clin. Investig. 106, 951–961
15. Hla, T., Lee, J. M., Ancellin, N., Kuks, K. J., and Kluk, M. J. (2001) Science 294, 1875–1878
16. Kohama, T. A., Olivera, A., Edsall, L., Nagie, M. M., Dickson, R., and Spiegel, S. (1998) J. Biol. Chem. 273, 23722–23728
17. Xia, P., Game, R., Rye, K. A., Wang, L., Hii, C. S. T., Cockerill, P., Khew-Goodall, Y., Bert, A. G., Barter, P. J., and Vadas, M. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 14196–14201
18. Melendez, A., Fito, R. A., Gillooly, D. J., Harrett, M. M., and Allen, J. M. (2000) J. Biol. Chem. 275, 147–155
19. Olivera, A., Kohama, T., Edsall, L., Nava, V. E., Cuvillier, O., Poulton, S., and Spiegel, S. (1999) J. Cell Biol. 141, 547–557
20. Ansell, N., Colmant, C., Su, J. L., Q. Mittereder, N., Chae, S.-S., Stafasson, S., Liu, G., and Hla, T. (2002) J. Biol. Chem. 277, 6667–6675
21. Kii, M., Wada, I., Imai, S., Sakane, F., and Kanoh, H. (1997) J. Biol. Chem. 272, 24572–24578
22. Priechel, E. E., Csonga, R., Novotny, V., Kikuchi, G., and Baumruker, T. (1999) J. Exp. Med. 190, 1–8
23. Im, D.-S., Clemens, J., Maldonald, T. L., and Lynch, K. R. (2001) Biochemistry 40, 14055–14060
24. Bolton, C., Borel, J. F., and Cuza, M. L. (1982) J. Biol. Chem. 257, 147–153
25. Kii, H., A. C., K., H., T., N., H., S., T., and S., S. (1998) J. Biol. Chem. 273, 1593–15920
26. Matsuda, S., Minowa, A., Suzuki, S., and Koyasu, S. (1999) J. Immunol. 162, 3321–3326
27. Nikolova, Z., Hof, A., Bauml, Y., and Hof, R. P. (2001) Transplantation 72, 168–171
28. Mandala, S. M., A., M., R., Galve-Roperh, I., Poulton, S., Peterson, C., and Gartner, A. (1996) Nature 381, 809–813
29. Miyamoto, T., Matsuomi, A., Hwang, M. W., Nishio, R., Ito, H., and Nishio, M. (2001) J. Am. Coll. Cardiol. 37, 1713–1718
30. Beer, M. S., A., M., J. A., A., R., S. (1998) Proc. Natl. Acad. Sci. U. S. A. 97, 7859–7864
31. Siegel, J., A., J., B., B., and T., T., (1999) J. Biol. Chem. 274, 118–129
32. Yamada, S., H., R., Bergström, J., Quackenberg, F., X., L., Milligan, L., Thorin, R., Shi, G. J., Card, D., Keeshan, C., Rosenbach, M., H., J., Lynch, C. L., Rupperrecht, K., Parsons, W., and Rosen, H. (2002) Science 296, 346–349
The Immune Modulator FTY720 Targets Sphingosine 1-Phosphate Receptors
Volker Brinkmann, Michael D. Davis, Christopher E. Heise, Rainer Albert, Sylvain Cottens, Robert Hof, Christian Bruns, Eva Prieschl, Thomas Baumruker, Peter Hiestand, Carolyn A. Foster, Markus Zollinger and Kevin R. Lynch

J. Biol. Chem. 2002, 277:21453-21457.
doi: 10.1074/jbc.C200176200 originally published online April 19, 2002

Access the most updated version of this article at doi: 10.1074/jbc.C200176200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 36 references, 18 of which can be accessed free at http://www.jbc.org/content/277/24/21453.full.html#ref-list-1