症後の投与のいずれによっても抑制されたが，対照に用いたα-GalCerではほとんど抑制されなかった[5]．また，再発型EAEもOCHの連続投与で抑制され，OCHを投与されたマウスでは再発はほとんどみられなくなった（未発表データ）．以上の実験結果は，OCHの連続投与によって自己免疫性の慢性炎症が制御できることを意味する．

4. ヒトNKT細胞はOCHを認識する

NKT細胞の糖脂質抗原認識については，TCR，CD1dともに種差を超えて高度に保存されており，その結果同一の糖脂質（α-GalCer）がマウスとヒトNKT細胞の両方に認識される[6-8]．しかし，OCHがヒトNKT細胞に本当に認識されるのか確認する必要があった．筆者らは最近，ヒト末梢血NKT細胞がOCHに反応して増殖することを証明し（荒木ら，論文準備中），OCHの臨床応用に期待を寄せている．また，末梢血から樹立したNKT細胞クローンによる解析も進めているが，OCHによるCD4$^+$NKT細胞のTh2偏倚誘導が最近確認できたところである．なお，この結果は，NKT細胞が単一クローンのレベルでも機能的な二面性を発揮することを示したものとしても重要である．

おわりに

MSのAPL療法は副作用（アレルギー反応，再発の誘導）のために頓挫したが，OCHで代表されるAGL療法（変換糖脂質療法）は実用化できる可能性が高い．その根拠として，NKT細胞の抗原認識システムがクローン間，個体間できわめて均一であり，APLのように一部の個体で質的に異なる危険な免疫応答を誘導する可能性が低いことがあげられる．OCHあるいは関連アナログのなかから一つでも良い性質を示すものが同定されれば，それを使って，すべての患者に等しく治療効果を及ぼすことが期待できる．糖脂質の反復投与によってNKT細胞が減少する，もしくは反応性が低下して治療効果が消えてしまうのではという懸念もあったが，関節炎モデルにおける連続長期投与の成功により払拭された．

これからの研究課題としては，lipid tailの長さが異なるだけで，なぜOCHとα-GalCerがこのように質的に異なる反応をNKT細胞に誘導するか解明することが重要である．基礎研究を進めることにより，OCHよりも優れたTh1自己免疫疾患治療薬や，まったく異なる作用をもつ糖脂質が開発できる可能性がある．NKT細胞を刺激するAGLは，今後自己免疫，アレルギー，癌免疫，免疫寛容などの広い分野で，有用な解析のツールとして，あるいは有効な治療薬として貢献することが期待される．

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▶自己免疫疾患におけるNKT細胞の数の減少，機能的変調について，$CD4^{-}$NKT細胞と$CD4^{+}$NKT細胞に分けて解析した最初の論文である．文献10-12）で発表された健常者の解析結果と対比すると興味深い．

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2. 自己免疫性脳脊髄疾患の糖脂質療法

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key words　NKT cell, glycolipid ligand, OCH, EAE, Th1/Th2

動　向

NKT細胞は，T細胞受容体（TCR）α鎖に可変性のないinvariant鎖（マウスではVα14Jα281，ヒトではVα24JαQ）を発現し，多型性のないCD1d分子により提示された糖脂質をリガンドとするユニークなリンパ球である[1-3]．TCRを介した刺激によりIL-4，IFN-γを短時間で大量に産生することから，その免疫調節機能が注目されている．自己免疫疾患においては，α-ガラクトシルセラミドやその誘導体であるOCHなどの糖脂質リガンドを用いて，実験的自己免疫性脳脊髄炎experimental autoimmune encephalomyeliti（EAE）を抑制できることが報告され[4-7]，自己免疫疾患の新しい治療方向として期待されている．

A. NKT細胞と多発性硬化症

NKT細胞は，多発性硬化症（MS）[8,9]，I型糖尿病（IDDM）[10,11]，進行性全身性硬化症[12]，慢性関節リウマチ，全身性エリテマトーデス，Sjögren症候群[13]などの自己免疫疾患の末梢血中での数が減少していることが報告されている．またこれらの全身性自己免疫性疾患では，NKT細胞の代表的な糖脂質リガンドであるα-ガラクトシルセラミド（α-GC）（図1）に対する反応が低下していることが報告され[13]，WilsonらはI型糖尿病から樹立したNKT細胞クローンは，IFN-γ産生に傾いていることなど機能的な異常を報告し，NKT細胞が自己免疫疾患病態に何らかの形で関与することが推測されている（表1）[10]．ただし，IDDMでは，NKT細胞数の減少が追試できないという報告があり[14,15]，また健常人のCD4CD8陰性NKT細胞（DN-NKT細胞）は通常IL-4を産生しない[16]にもかかわらず，Wilsonらの報告ではIL-4が検出されていることなどから，彼等の結果が疑問視されるなど渾沌とした状況になっている．

MSでは，荒木・山村らが寛解期にあるMS患者では，健常人と比較して減少しているが，再発期にはNKT細胞の減少はむしろ軽度であることを報告している[9]．この際，減少しているのはDN-NKT細胞であり，$CD4^+$ NKT細胞は寛解期，再発時ともに減少していなかった．サイトカイン産生に関しては，DN NKT細胞では寛解期にIL-4，IFN-γともに産生の低下がみられたが，$CD4^+$ NKT細胞では寛解期にむしろIL-4の産生亢進がみられた．実際に，自己免疫疾患においてNKT細胞がどのような機能を果たしているかについては不明であるが，MS寛解期にはIFN-γなどのサ

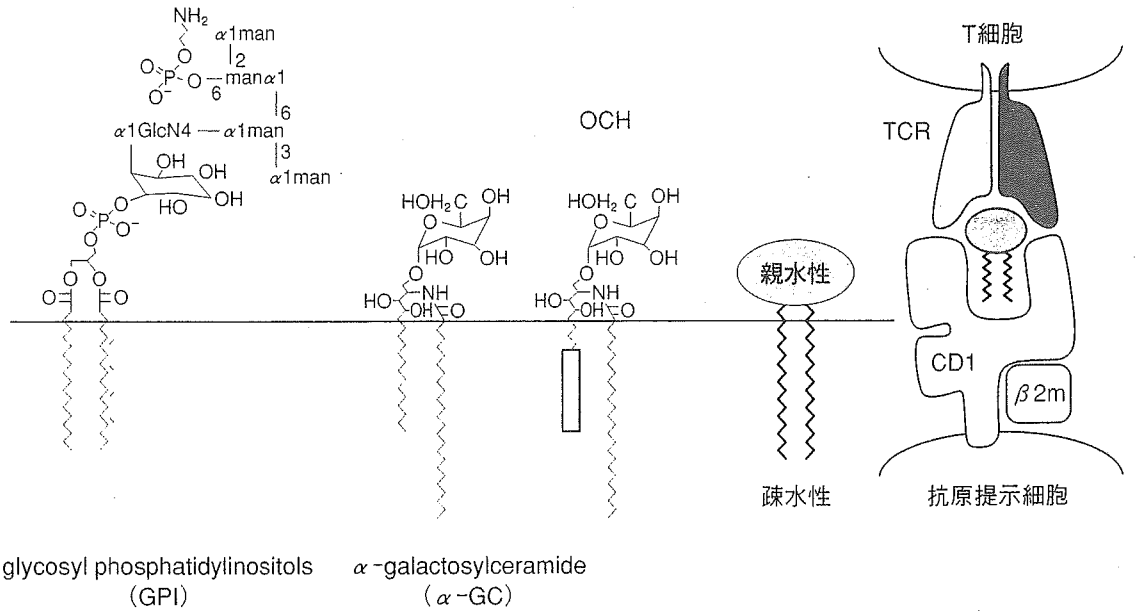

図1　NKT細胞のリガンド（文献2からの改変）
NKT細胞のリガンドとしては，α-GCが知られているが，他にGPIもその候補として報告されている．これらの糖脂質は，親水性の糖鎖と疎水性の脂質部分からなり，疎水性の部分がCD1の疎水性のポケットに入ることが想定される．OCHは，α-GCのスフィンゴシン鎖を短縮した合成体である．

イトカインを産生するDN-NKT細胞数が減少し，DN-NKT細胞から産生されるサイトカインも減少し，残存しているCD4$^+$NKT細胞のIL-4産生能があがっていることを考えると，MS寛解期においてNKT細胞は疾患を抑制するように働いていることが推定される．

表1　ヒト自己免疫疾患とNKT細胞

	文献
数の異常	
多発性硬化症	8，9)
I型糖尿病	10，11，14，15)
全身性強皮症	12)
全身性エリテマトーデス	13)
関節リウマチ	13)
Sjögren症候群	13)
機能変化	
多発性硬化症	9)
I型糖尿病	10)
全身性強皮症	13)
全身性エリテマトーデス	13)
慢性関節リウマチ	13)
Sjögren症候群	13)

自己免疫疾患の動物モデルにおいても，全身性エリテマトーデスの自然発症モデルであるMRL lpr/lprやNZB/WF1，I型糖尿病の自然発症モデルであるNODマウスなどでNKT細胞が減少していることが報告されている[17,18]（表2）．特にNODマウスでは，NKT細胞の移入や，Vα14-Jα281T細胞受容体遺伝子によりNKT細胞を増加させると糖尿病発症を阻止することが示されており，自己免疫病態に関与していることが明らかになっている[19,20]．さらに，MSのモデルであるEAE[4-6]やNODにおける糖尿病発症は，NKT細胞の糖脂質リガンドの投与によって抑制されることが報告され[21-23]，自己免疫疾患の糖脂質療法が注目されている．そこで，まずこれまで報告されているNKT細胞の糖脂質リガンドについて紹介する．

B. NKT細胞と糖脂質抗原

NKT細胞は，半可変性のT細胞受容体を発現

表2　自己免疫疾患モデルとNKT細胞

	文献
実験的自己免疫性脳脊髄炎	
OCHによる病態抑制	4)
α-GCによる病態抑制	5～7)
NODマウスにおける糖尿病	
NKT細胞の数の減少	17, 29)
DN-NKT細胞移入による糖尿病抑制	18)
Vα14Jα281トランスジェニックマウスにおける糖尿病抑制	19)
CD1ノックアウトマウスにおける糖尿病悪化	21, 30)
α-GCによる糖尿病抑制	21～23)
ループスモデル	
MRL *lpr/lpr*, C57BL/6 *lpr/lpr*, (NZBxNZW) F1, C3H*gld/gld*におけるNKT細胞の数の減少	18)
(NZBxNZW) F1における抗CD1抗体による病態改善	31)
炎症性腸炎	
α-GCによる病態抑制	32)

し，CD1分子によって提示される糖脂質抗原を認識する．CD1分子はβ2マイクログロブリンと非共有結合したヘテロ2量体として細胞表面に発現し，MHCクラスI分子に類似している[2]．CD1ファミリーには，ヒトではグループ1-CD1（CD1a, b, c）とグループ2-CD1（CD1d）が知られているが，マウスではCD1d分子のみが存在する．CD1分子は，MHC分子と異なり多様性がないため，同一種内では共通である．CD1d分子の結晶構造解析では，MHCクラスI分子に類似するが，抗原結合溝はきわめて疎水性である．抗原結合溝が疎水性であること，NKT細胞の発生が抗原関連トランスポーター（TAP）非依存性であること，他のCD1ファミリー分子が結核菌細胞壁の構成脂質であるミコール酸やレプラ菌の細胞壁糖脂質であるリポアラビノマンナン（LAM）を抗原提示することなどから，CD1dに抗原提示を受けるNKT細胞も，蛋白ではなく糖脂質をリガンドとして認識すると考えられた[1-3]．直接的な証明としては，NKT細胞の抗原として，谷口らが海綿の成分であるα-ガラクトシルセラミド（α-GC）がNKT細胞の抗原となることを発見したことであるが[24]，α-GCは哺乳類の生体内には存在しないので，ナチュラルリガンドについてはまだ不明な点が多い．ヒトの臍帯血中のNKT細胞や，germ freeマウスのNKT細胞もすでにメモリーマーカーが陽性であることから，何らかの自己抗原を認識しているのではないかと考えられている．JoyceらはCD1分子にglycosylphosphatidylinositol（GPI）が結合していることを報告している[25]（図1）が，GPIが生体内でのリガンドなのかどうかについては，まだ異論も多く，結論は出ていない．

現在NKT細胞の活性化に最もよく用いられているのはα-GCであるが，α-GCはNKT細胞を刺激して，IL-4，IFN-γを産生させる．MSをはじめとする臓器特異的自己免疫疾患は，多くの場合IL-4などのTh2サイトカインが疾患抑制的に作用し，IFN-γなどのTh1サイトカインは疾患を増悪させると考えられている．そこで，我々はNKT細胞を刺激して疾患抑制的なTh2サイトカインを選択的に産生させることができる糖脂質を探索し，α-GCの誘導体の一つ（OCH）がこのような性質をもつことがわかった（図2）[4]．OCHはα-GCのスフィンゴシン鎖を短くした誘導体である（図1）．C57BL/6（B6）マウスにα-GCを

投与すると，IL-4だけでなくIFN-γも上昇するが，OCHを投与すると，血清中のIL-4が選択的に上昇する．NKTノックアウトマウスでは，OCHやα-GC投与による血中サイトカインの上昇はみられないことから，NKT細胞を介するものであることがわかった．α-GCはごく低容量でもIFN-γとIL-4の両方を産生させることから，OCHとα-GCが与えるシグナルどのような違いがあるのか興味深い．構造的に考えると，OCHはα-GCのスフィンゴシン鎖が短縮されており，CD1dとの結合安定性がα-GCに比較して弱いことが推定されるので，NKT細胞の刺激時間が短くなるという可能性がある．もう一つの可能性は，CD1dとOCHの結合が，α-GCと若干異なりT細胞に認識される糖部分が3次元的に異なって提示されているということであるが，詳細については結晶構造解析など今後の研究が待たれる．

C. 実験的自己免疫性脳脊髄炎における糖脂質療法

EAEは，MSの動物モデルで，Th1細胞が介在する自己免疫疾患モデルである．我々はNKT細胞を活性化することによってEAEが抑制されることを期待して，まずNKT細胞の代表的な糖脂質リガンドであるα-GCのEAEに対する効果を

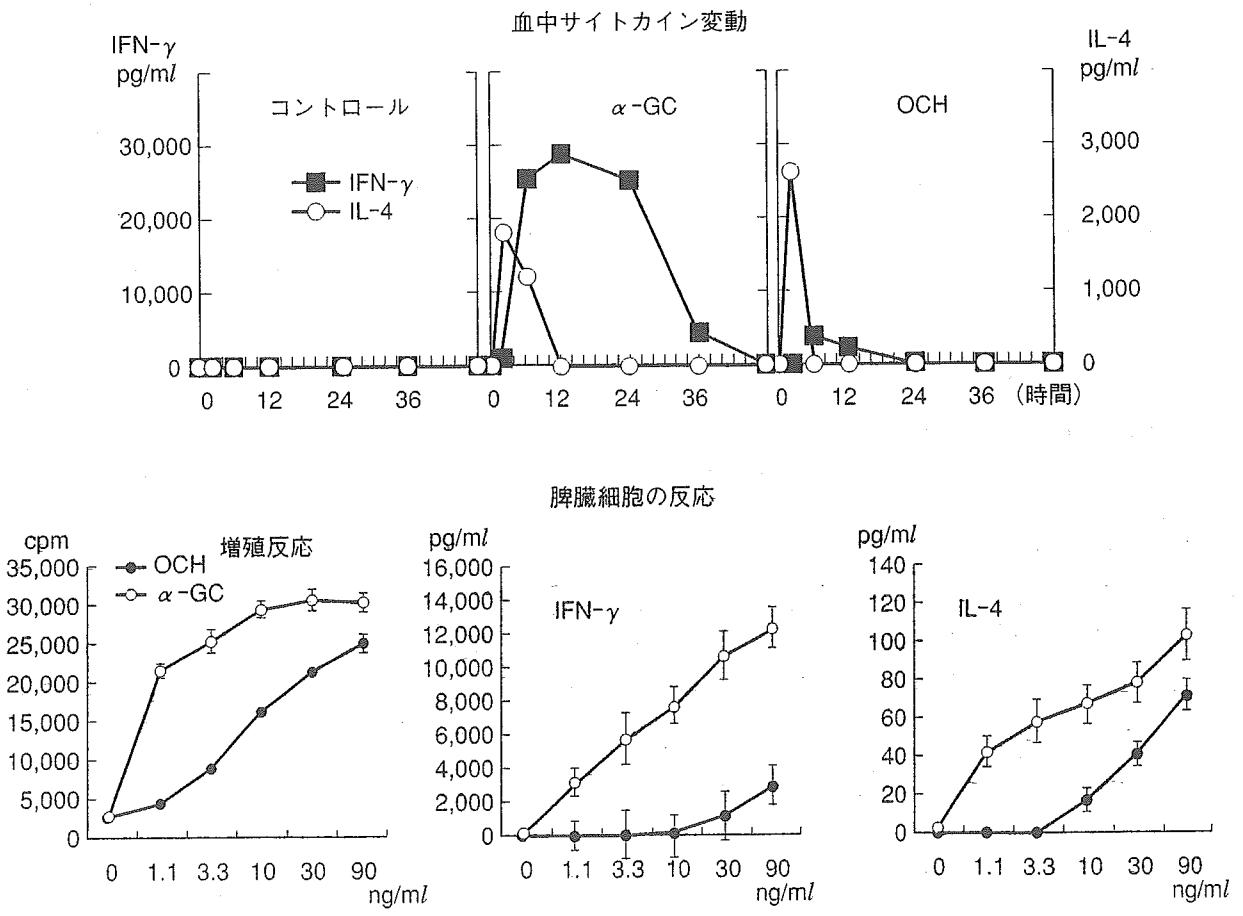

図2　OCH，α-GCのin vivo，in vitroでのサイトカイン誘導能の違い（文献3からの改変）

上段: α-GCもしくは，OCHをB6マウスに投与後，時間経過を追って血中のIFN-γ，IL-4をELISAで測定した．

下段: α-GCもしくはOCHをB6マウス脾臓細胞に加え48時間培養し，培養液中のIFN-γ，IL-4をELISAで測定した．α-GC投与ではIFN-γ，IL-4がともに上昇するが，OCH投与では，IL-4が選択的に上昇することがわかった．

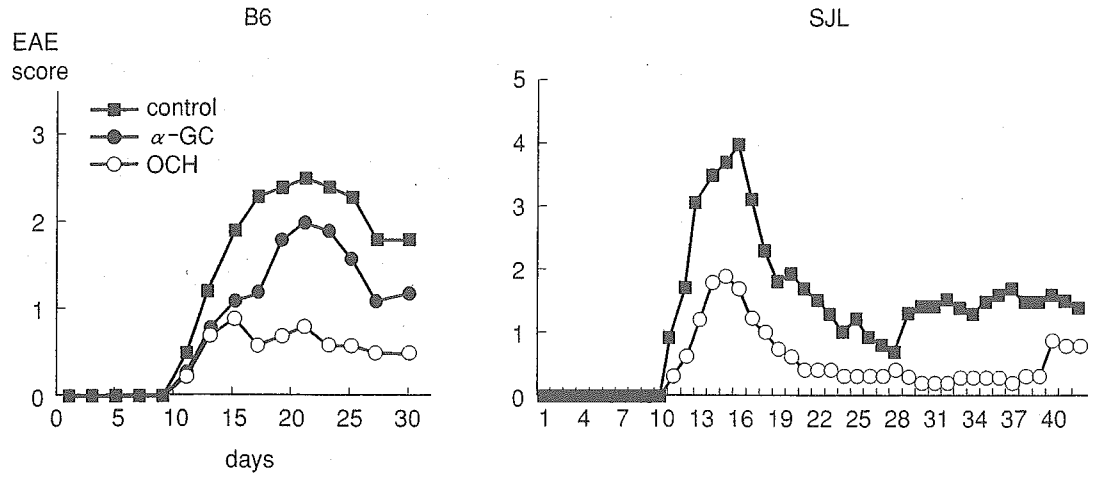

図3　糖脂質によるEAE抑制効果

C57BL/6（左），SJLマウス（右）におのおのMOG$_{35-55}$もしくはPLP$_{139-151}$を免疫し，EAEを誘導した．OCHもしくはα-GCを抗原感作時（左）もしくは，感作4日後（右）より投与して疾患抑制効果を検討した．

検討した．しかし，EAEはα-GCによって何ら抑制を受けなかった[26]．IL-4，IFN-γなどのノックアウトマウスなどを使った一連の解析の結果，α-GCがEAEに無効である理由は，NKT細胞にTh1抑制的なIL-4だけでなくTh1促進的なIFN-γの産生をうながすためであると考えられた．そこで，NKT細胞にIL-4だけを産生させることができれば，EAEは抑制できると考え，NKT細胞に選択的にIL-4産生をうながしてEAEを抑制するような方法を検討した．B7.2などの副刺激を抗体で抑制した状態でα-GCをパルスした抗原提示細胞を移入すると，NKT細胞はIL-4を優位に産生し（Th2偏倚），EAEの発症も予防できることがわかった[26]．しかしながら臨床応用を考えると細胞移入という手法はやや煩雑であるため，NKT細胞をTh2偏倚させる糖脂質リガンドの探索を試み，α-GCの誘導体の一つ（OCH）がこのような性質をもつことがわかった（図2）[3]．その選択的なIL-4誘導能に一致して，OCHをEAE誘導時に経口投与するとEAEは強く抑制された．また，OCH投与群では免疫源であるMOGペプチド$_{35-55}$に対する抗体のアイソタイプを測定すると，抗MOG$_{35-55}$ IgG1が選択的に上昇しており，MOG$_{35-55}$に対する自己免疫応答がOCHによりTh2に偏倚していた．さらに，OCHの作用がIL-4を介するかどうか検討するために，抗IL-4抗体による中和実験とIL-4ノックアウトマウスを使った実験を行った．その結果，OCHと抗IL-4抗体を同時投与するとEAEの抑制はみられなくなった．IL-4ノックアウトマウスではOCHのEAE抑制効果はみられず，OCHのEAE抑制にはIL-4が重要な役割を果たすことが明らかになった．

その後α-GCの投与でEAEが抑制されるということが報告された[5,6]．これらのグループは，EAE誘導時の1回腹腔投与，誘導に抗原と同時にエマルジョンとして皮下投与，誘導1週間前に投与するなどの方法で，B6マウスに誘導されたEAEに効果があることを報告した．我々は，α-GCの投与では，いずれの方法でもEAEを抑制することができなかった．最近，さらに別のグループからEAE誘導時に抗原と同時にエマルジョンとして皮下投与すれば有効だが，腹腔内投与は無効であったという報告がなされた[7]．α-GCについては前述のように様々な報告があるが，これらの結果の違いについての理由は不明である．これまでもB6マウスにα-GCを投与することによって，

Th2応答を誘導できるという報告と[27]，むしろIgE低下がみられ，Th1応答に傾くという報告[28]がある状況と類似している．NKT細胞はIL-4とIFN-γという機能的には相反するサイトカインを同時に産生する能力があることから，Th1/Th2分化への影響にはその微妙なバランスが重要なのであろう．いずれにしても抗原と同時にエマルジョンとして皮下投与するのは，臨床応用を考えると現実的ではない．

OCHは，EAEばかりでなく，コラーゲン関節炎や，NODマウスにおける糖尿病発症も抑制することが明らかとなっており（千葉，三宅，未発表），OCHは広くTh1病に効果がある．NODマウスにおいては，従来の報告のように，α-GCの投与でも，糖尿病の発症は抑制されたが，関節炎ではα-GCの効果はみられていない．α-GCは，NODでは効果がはっきりみられるが，他の自己免疫モデルでは効果がまちまちで，自己免疫治療としてはOCHの方が用途が広いようだ．

D. MS治療への臨床応用の可能性

これまで，OCHがTh1タイプの臓器特異的自己免疫疾患モデルには有効であることを紹介したが，OCHのような糖脂質が実際に臨床応用できるであろうか？

まず，自己免疫疾患ではNKT細胞数が減少していると報告されているが，このような状態で，NTK細胞の刺激は有効だろうか．これについては，NKT細胞の数が少ないSJLマウスにおいても，OCHはEAEを抑制し，さらに再発も抑制していること，またSJLマウスに誘発したコラーゲン関節炎も抑制していること，NTK細胞数の少ない別の系統であるNODマウスで糖尿病発症を抑制していることなどから，NKT細胞数が減少している状態でも効果は期待できると思われる．

次に，糖脂質を人体に投与することの安全性であるが，α-GCは抗癌剤として治験が開始されており，Phase Iでは安全性が確認されている．OCHについてはまだ人体への投与は検討されていないが，マウスやラットにおいて，α-GCより強い毒性などはみられていない．

ヒトNKT細胞が，OCHに反応するかどうかということも重要な問題である．末梢血から樹立したNKTクローンを用いた実験では，OCHにはCD4$^+$NKT細胞が特異的に反応し，α-GC刺激と比較すると，Th2サイトカインを有意に高く産生することが確認でき，マウスNKT細胞と類似した効果が期待できる（荒木，未発表）．

むすび

NKT細胞は，様々な自己免疫疾患や自己免疫モデルで数や機能の異常が指摘されており，自己免疫疾患病態に関与していると考えられる．抗原特異的治療が自己免疫疾患治療の理想であるが，抗原の多様性，抗原提示するMHC側の多様性，ペプチドに対するアレルギー反応など実現には克服すべき問題も多いことがわかってきた．自己免疫疾患のような，緩解と増悪を繰り返し，免疫調節の微妙なバランスの上に成り立っているような病態では，NKT細胞のような免疫調節細胞を治療標的とすることも一つの新たな方向性であると思われる．

糖脂質リガンドは，医薬としてみた場合，ペプチド治療の際に問題となる，抗原提示分子の多型性を考慮する必要のないことなどから，広くTh1細胞の関与する自己免疫疾患に応用できる治療薬となることが期待できる．

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Neuropeptide Y (NPY) Suppresses Experimental Autoimmune Encephalomyelitis: NPY_1 Receptor-Specific Inhibition of Autoreactive Th1 Responses In Vivo[1]

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Prior studies have revealed that the sympathetic nervous system regulates the clinical and pathological manifestations of experimental autoimmune encephalomyelitis (EAE), an autoimmune disease model mediated by Th1 T cells. Although the regulatory role of catecholamines has been indicated in the previous works, it remained possible that other sympathetic neurotransmitters like neuropeptide Y (NPY) may also be involved in the regulation of EAE. Here we examined the effect of NPY and NPY receptor subtype-specific compounds on EAE, actively induced with myelin oligodendrocyte glycoprotein 35–55 in C57BL/6 mice. Our results revealed that exogenous NPY as well as NPY Y_1 receptor agonists significantly inhibited the induction of EAE, whereas a Y_5 receptor agonist or a combined treatment of NPY with a Y_1 receptor antagonist did not inhibit signs of EAE. These results indicate that the suppression of EAE by NPY is mediated via Y_1 receptors. Furthermore, treatment with the Y_1 receptor antagonist induced a significantly earlier onset of EAE, indicating a protective role of endogenous NPY in the induction phase of EAE. We also revealed a significant inhibition of myelin oligodendrocyte glycoprotein 35–55-specific Th1 response as well as a Th2 bias of the autoimmune T cells in mice treated with the Y_1 receptor agonist. Ex vivo analysis further demonstrated that autoimmune T cells are directly affected by NPY via Y_1 receptors. Taken together, we conclude that NPY is a potent immunomodulator involved in the regulation of the Th1-mediated autoimmune disease EAE. *The Journal of Immunology,* **2003, 171: 3451–3458.**

Experimental autoimmune encephalomyelitis (EAE)[3] is an animal autoimmune disease that can be induced with sensitization against CNS components such as myelin oligodendrocyte glycoprotein (MOG) (1, 2). Because the neurological signs of paralysis can be monitored continuously and because the pathological findings characterized by focal mononuclear cell infiltrates and demyelinating lesions resemble those found in multiple sclerosis (MS), this representative autoimmune disease model is widely used. It is established that EAE is mediated by $CD4^+$ Th1 T cells producing IFN-γ and TNF-α in response to the peptide of the CNS components. In support of this consensus, a number of studies have proven that polarizing autoimmune Th1 cells toward Th2 directions (3–7) leads to suppression of the clinical and pathological manifestations of EAE. These findings indicate that human Th1-mediated diseases such as MS could also be treated or prevented with the Th2-inducing protocols effective in suppression of EAE. Thus, molecular mechanisms controlling the Th1/Th2 balance needs to be further elucidated in terms of the regulation of autoimmunity.

It is now well established that the immune system and the nervous system are connected bidirectionally (8–10). Although much remains to be investigated, several lines of evidence suggest that the sympathetic nervous system (SNS) provides a major pathway for neuroimmune interactions. Indeed, a role for catecholamines such as norepinephrine and epinephrine in SNS-mediated immunoregulation has been implicated in various conditions (11–15). Regarding the modulation of autoimmunity, it was previously demonstrated that depletion of SNS transmitters by chemical sympathectomy enhances the severity of EAE (11, 12). Because β-adrenoceptor agonists protect against EAE (13) and catecholamines modulate several immunological functions critical to the pathogenesis of EAE (14), the enhancement of EAE by chemical sympathectomy has largely been attributed to the depletion of catecholamines. However, although neuropeptide Y (NPY) is also released from SNS terminals innervating lymphatic tissues (16, 17), no previous studies have explored the possibility that depletion of other SNS transmitters such as NPY may contribute to these findings.

NPY is a 36-aa peptide. This amidated peptide is abundant in neurons and can be detected in all parts of the body. NPY regulates a variety of physiological activities, including energy balance and feeding, anxiety, neuroendocrine secretion, neuronal excitability, and vasoconstriction (18, 19). NPY exerts its pleiotropic functions through the activation of several G-protein coupled NPY receptor subtypes (18). Accumulating evidence indicates that NPY receptor subtypes mediate the differential actions of NPY (18) and that they are differentially expressed in the mammalian tissues. Whereas expression of Y_2 and Y_5 receptor is highly restricted to the CNS,

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[3] Abbreviations used in this paper: EAE: experimental autoimmune encephalomyelitis; LN, lymph node; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NPY, neuropeptide Y; PLP, proteolipid protein; SNS, sympathetic nervous system.

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Y_4 receptors are selectively expressed in the periphery. In contrast, Y_1 receptors are rather ubiquitously expressed; their presence has been reported in brain, heart, kidney, gastrointestinal tract, endothelial cells, and leukocytes (18, 19).

Of note, NPY can be found in the storage vesicles of the sympathetic nerve terminals innervating lymph nodes, spleens, and the bone marrow of various species (19). Furthermore, Y_1 receptors were demonstrated on rat PBMC (20, 21). These results suggested a role for NPY in neuroimmune interactions. In support of this hypothesis, two independent studies previously showed that NPY significantly modifies the cytokine profile of T helper clone cells in vitro (22, 23). Namely, Levite (23) reported NPY converts the cytokine profile of Th1 clones to a Th0 type in vitro, whereas Kawamura et al. (22) showed that NPY inhibits the IFN-γ production by Th1 clones as well as that of freshly isolated spleen T cells. However, despite the potential of NPY to induce a Th2 shift in vitro, it remains unclear whether NPY may alter the cytokine profile of Th1 cells in vivo. This prompted us to investigate a possible role of NPY in the regulation of EAE mediated by Th1 cells.

To explore the role of NPY in vivo, we immunized female C57BL/6 (B6) mice with MOG_{35-55} and treated them with NPY and/or NPY receptor subtype-selective compounds every other day. Here we report that exogenous NPY significantly suppresses the clinical course of EAE and that this effect is mediated through the activation of Y_1 receptors expressed by T cells. Our experiments have revealed that suppression of IFN-γ production by MOG_{35-55}-specific Th1 cells and the concomitant Th2 bias account for the suppression of EAE.

Materials and Methods

Mice and reagents

Female B6 mice were purchased from CLEA Laboratory Animals (Tokyo, Japan), and female SJL/J mice were purchased from Charles River Japan (Tokyo, Japan). The animals were kept under specific pathogen-free conditions and were subjected to experiments at 6–10 wk of age. Rat MOG_{35-55} (amino acid sequence, MEVGWYRSPFSRVVHLYRNGK) was synthesized at Chiron Technologies (Clayton, Victoria, Australia), and proteolipid protein (PLP) 139–151 (amino acid sequence, HCLGKWL GHPDKF) at Toray Research Center (Tokyo, Japan). IFA and heat-killed *Mycobacterium tuberculosis* H37Ra were obtained from Difco (Detroit, MI), and pertussis toxin was obtained from List Biological Laboratories (Campbell, CA). NPY was purchased from Sigma-Aldrich (St. Louis, MO). A Y_1 receptor agonist, [F^7,P^{34}]NPY, and a Y_5 receptor agonist, [Ala^{31},Aib^{32}]NPY, were generated as previously described (24, 25). Another Y_1 receptor agonist, [D-His^{26}]NPY (26), was a gift from Schering (Kenilworth, NJ). Receptor specificity of these compounds was achieved by replacing certain amino acids at specific positions that are critical for the structural interaction of native NPY with different NPY receptor subtypes (for details see Table I). The Y_1 receptor antagonist BIBO3304, a small nonpeptide compound (27), was kindly provided by Boehringer Ingelheim (Biberach, Germany).

Immunization

Active EAE was induced in B6 mice as described previously (6, 7). Briefly, the mice were challenged in the tail base with an emulsion containing 100 μg of MOG_{35-55} and 500 μg of *M. tuberculosis* in IFA. Directly after the immunization and 48 h later, the mice were injected i.p. with 500 ng of pertussis toxin. SJL/J mice were immunized s.c. with an emulsion containing 100 μg of $PLP_{139-151}$ and 1000 μg of *M. tuberculosis* in IFA. They were injected with 200 ng of pertussis toxin shortly after immunization.

Clinical assessment

Mice were observed daily for clinical signs of EAE. Disease severity was scored and evaluated as follows: 0 = normal; 1 = weakness of the tail and/or paralysis of the distal half of the tail; 2 = loss of tail tonicity; 3 = partial hind limb paralysis; 4 = complete hind limb paralysis; 5 = forelimb paralysis or moribund; 6 = death. Cumulative scores were calculated for an individual mouse by summing up the daily scores.

Application of NPY and NPY receptor subtype-specific compounds

NPY and the receptor subtype-specific compounds were diluted in PBS. The animals were injected every second day with NPY and/or these compounds throughout the experiment, unless otherwise stated. Control mice were injected with 200 μl of PBS on alternate days. To treat mice with a combination of NPY and the Y_1 receptor antagonist BIBO3304 on alternate days, we injected NPY and the antagonist on the same day (NPY injection followed by BIBO3304) and gave two injections of PBS to control mice.

Measurement of MOG_{35-55}-specific IgG1 and IgG2a titers

ELISA plates were coated with 10 μg/ml MOG_{35-55} in PBS overnight at 4°C. After blocking with 3% BSA in PBS, serial dilutions of the serum from animals at day 40 after immunization, or normal mice or PBS were added to the plates. MOG_{35-55}-specific Abs were detected, using biotin-labeled anti-IgG1 and anti-IgG2a Abs. After adding streptavidin-peroxidase and a substrate, Ab concentrations were estimated on the basis of dilutions/OD curves.

MOG_{35-55}-specific T cell proliferation assay

After immunization with MOG_{35-55}, the animals were treated every second day with the indicated compounds from day 0 to day 10 after immunization. The mice were sacrificed at day 10 and inguinal and popliteal lymph nodes (LN) were removed. Total LN cells were suspended in RPMI 1640 supplemented with 5×10^{-5} M 2-ME, 2 mM L-glutamine, 100 U/100 mg/ml penicillin/streptomycin, and 1% syngeneic mouse serum (standard medium). We incubated the cells in 96-well round-bottom plates at 1×10^6/well for 72 h (37°C, 5% CO_2 atmosphere) in the presence of MOG_{35-55} (1, 10, or 100 μg/ml). Incorporation of [^{3}H]thymidine (1 μCi/well) for the final 16 h of the culture was determined with a β-1205 counter (Pharmacia, Uppsala, Sweden).

To determine whether the suppressive effects of a Y_1 receptor agonist, [D-His^{26}]NPY, are due to its interaction with T cells or with APC, T cells were isolated from the LN using a standard nylon wool column procedure. The LN cells were obtained from MOG_{35-55}-primed mice treated with [D-His^{26}]NPY or PBS. They were applied to the nylon wool column and incubated for 1 h at 37°C (5% CO_2 atmosphere), and the T cells were harvested from the column by gently rinsing with RPMI 1640 containing 5% FCS. The LN cells that had been x-irradiated with 4000 rad were used

Table I. *Amino acid sequence of NPY and receptor subtype-specific NPY analogs*

Peptide	Preference	Amino Acid Sequence	Alteration	Reference
NPY	Y_{1-6} receptor	YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY		
[F^7,P^{34}]NPY	Y_1 receptor	YPSKPD**F**PGEDAPAEDLARYYSALRHYINLITR**P**RY[a]	Amino acids in positions 7 and 34 are replaced	24
[D-His^{26}]NPY	Y_1 receptor	YPSKPDNPGEDAPAEDLARYYSALR**H**YINLITRQRY	L-His in position 26 is replaced by D-His	26
[Ala^{31},Aib^{32}]NPY	Y_5 receptor	YPSKPDNPGEDAPAEDLARYYSALRHYINL**AB**RQRY	Amino acids in positions 31 and 34 are replaced by a synthetic dipeptide	25

[a] Replaced amino acid residues are bold and underlined as shown.

as APC. T cells (5×10^5/well) and APC (5×10^5/well) were than cocultured in 96-well round-bottom plates in the presence or absence of MOG_{35-55}. Cytokine assay was conducted as described below for the supernatants harvested at 48 h. Cell proliferation was determined by measuring the incorporation of [^{3}H]thymidine (1 μCi/well) in the final 16 h of 72-h cultures.

Cytokine assay

To evaluate the effect of Y_1 receptor stimulation on the cytokine secretion, LN cells from the MOG_{35-55}-immunized, NPY-treated mice were suspended in the standard medium and cultured in 96-well round-bottom plates at 1×10^6/well for 48 h in the presence of MOG_{35-55}. The concentrations of IFN-γ and IL-4 in the supernatants were determined by using a sandwich ELISA. The assays were performed according to the protocol provided by BD PharMingen (San Diego, CA). All the reagents, including recombinant mouse cytokines and Abs, were purchased from BD PharMingen.

Anti-CD3 stimulation of splenocytes derived from naive mice

For the stimulation of the Ag receptor complex of T cells, 96-well round-bottom plates were coated with 1 μg/ml anti-CD3 mAb (clone 2C11) (BD PharMingen) overnight. After three washings with PBS, splenocytes (1×10^6/well) from untreated, naive animals were added and incubated in the standard medium for 48 h in the presence of various concentrations of [D-His^{26}]NPY. IFN-γ levels in the supernatants were detected with the sandwich ELISA.

Induction of passive EAE in SJL/J mice

At day 10 after immunization with $PLP_{139-151}$, the total spleen and draining LN cells were prepared from the mice and stimulated with the PLP peptide (30 μg/ml) in the standard medium. The cells were harvested 96 h after culture, and 1.6×10^7 of the cells were injected i.p. into each recipient that had been x-irradiated (300 rad) shortly before cell transfer. The recipient mice were further treated with pertussis toxin on the day of cell transfer and 2 days later (200 ng for each i.p. injection).

In vitro T helper cell differentiation

Spleen T helper cells were polarized for either Th1 or Th2 direction according to the protocol described by others (28). In brief, CD4$^+$CD44^{low} naive T cells were purified from the spleen of young B6 mice by using the magnetic beads (Dynal, Oslo, Norway), and the cells were stimulated with anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) under Th1- or Th2-inducing conditions. Namely, Th1 cells were induced in the presence of mouse IL-12 (5 ng/ml) and anti-IL-4 mAb (HB188; 10 μg/ml), whereas Th2 cells were induced in the presence of mouse IL-4 (1000 U/ml), anti-IFN-γ (HB170; 5 μg/ml), and anti-IL-12 (3 μg/ml). Three days later, the cells were fed with the fresh medium supplemented with 100 U/ml IL-2 in addition to the cytokines and Abs used in the primary stimulation. Eight days later, the cells were harvested and subjected to RNA preparation.

RT-PCR and real time PCR

RT-PCR was used to determine the transcription level of NPY Y_1 receptor in the LN cells from MOG_{35-55}-sensitized animals or in the nylon wool-purified spleen T cells from naive mice. Homogenized brain tissues from naive mice served as controls. Total RNA was extracted from these samples using RNABee (Tel-Test, Friendswood, TX). RNA (5 μg) was subjected to reverse transcription with the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA), and 35 cycles of PCR were conducted using *Taq*DNA polymerase and GeneAmp PCR system 9700 (Perkin-Elmer, Applied Biosystems, MA). Each cycle of PCR amplification comprised denaturation (95°C for 5 min), annealing (54°C for 30 s), and amplification (72°C for 60 s). The products of these reactions were analyzed by 2% gel electrophoresis. Primers used were as follows: Y_1 receptor sense, CTTCGGGGAGACCATGTGCAAACTGAATC; Y_1 receptor antisense, AGGAGAGTCGTGTAAGACAG; GAPDH sense, AACGACC CCTTCATTGAC; GAPDH antisense, TTCACGACATACTCAGCAC. Real time PCR was conducted by using the Light Cycler quantitative PCR system (Roche Molecular Biochemicals, Mannheim, Germany). We used a commercial kit (Light Cycler-FastStart DNA Master SYBR Green I; Roche Molecular Biochemicals) according to the manufacturer's instructions.

Statistical analysis

We used the Mann-Whitney test to analyze the differences in the clinical score of treatment vs control group. Data for cytokines and proliferative responses were subjected to overall two-way ANOVA. When there was a significant difference, a Fisher post hoc test was implemented. The statistical analysis was performed using SPSS for Windows.

Results

NPY inhibits actively induced EAE in a dose-dependent manner

To investigate a possible effect of NPY on actively induced EAE, we immunized female B6 mice with MOG_{35-55} to actively induce EAE. The mice were injected with 0.01–50 μg/kg NPY i.p. on alternate days from the day of immunization (day 0) until the termination of the experiments. The selection of NPY dosages is based on previous studies (29). We found that the continuous, alternate day treatment with NPY inhibits the clinical severity of EAE in a dose-dependent manner (Fig. 1). The maximum disease score was significantly inhibited when the mice were treated with 50 μg/kg (but not 0.01 or 1.0 μg/kg) of NPY (Fig. 1*A* and Table II). However, the cumulative disease score was effectively suppressed at both 1 and 50 μg/kg (Fig. 1*B*).

The inhibitory action of NPY is due to Y_1 receptor activation in vivo

Given that differential actions of NPY are mediated through distinct receptor subtypes (18, 19), we sought to elucidate which NPY receptor subtypes are involved in the EAE-inhibitory action of NPY. To this aim, we used various NPY receptor subtype-specific compounds. Lymphoid cell expression of Y_1 receptor has been

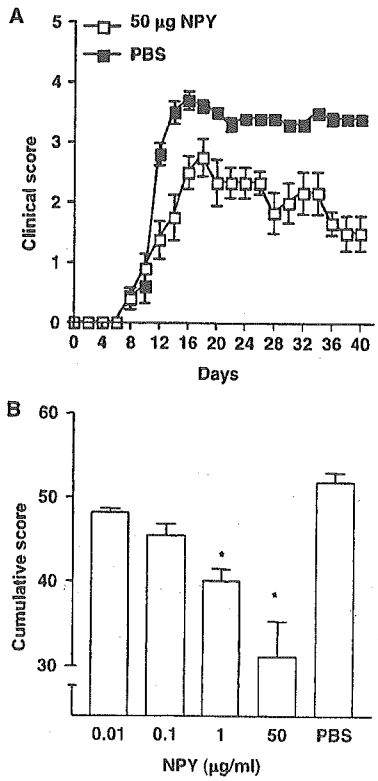

FIGURE 1. Effect of NPY on actively induced EAE. EAE was induced in female B6 mice by an immunization with MOG_{35-55} in CFA as described in *Materials and Methods*. *A*, Repetitive treatment with NPY (50 μg/kg) suppresses the clinical course of EAE as compared with sham-treated animals (PBS). *B*, Treatment with various NPY dosages induced a dose-dependent inhibition of EAE as assessed by cumulative disease scores. Statistical analysis reveals a significant inhibition of the cumulative disease score at 1 μg/kg ($p = 0.0196$) and 50 μg/kg ($p = 0.0176$) vs control mice injected with PBS. One representative experiment is shown ($n = 5$ for each group) and data are expressed as mean ± SEM. *, Significant differences between NPY and controls (PBS). Further statistical analysis is shown in Table II.

Table II. *Effect of NPY/receptor-specific analogs on EAE actively induced in wild-type B6*[a]

Treatment	Day of Onset	Maximum Score	Cumulative Score
NPY, 50 μg/kg	10.7 ± 2.67	2.67 ± 0.34* (0.001)[b]	31.2 ± 7.23* (0.006)
[D-His^{26}]NPY (Y_1R agonist), 0.01 μg/kg	13.0 ± 0.58	2.87 ± 1.03	30.8 ± 7.71* (0.032)
[F^7,P^{34}]NPY (Y_1R agonist), 1.0 μg/kg	13.2 ± 1.00	3.00 ± 0.50 (0.032)	35.3 ± 7.28* (0.032)
BIBO 3304 (Y_1R antagonist), 100 μg/kg	8.5 ± 0.50* (0.032)	3.88 ± 0.32	52.5 ± 7.51
[Ala^{31},Aib^{32}]NPY (Y_5R agonist), 50 μg/kg	10.8 ± 0.49	3.90 ± 0.10	52.2 ± 1.82
Control (PBS)	11.2 ± 0.80	4.20 ± 0.20	54.5 ± 1.24

[a] Representative experiments in wild-type B6 mice treated with NPY and various receptor-specific analogs. Experimental procedures are described in *Materials and Methods*. Data represent mean ± SEM at day 40 of the total mice in each group (n = 5). Significant differences between NPY/analog and controls (PBS) are indicated by asterisks (Mann-Whitney test).
[b] Numbers in parentheses are p values.

recently reported (20, 21). In a first step, we treated MOG_{35-55}-immunized mice with a combination of the amount of NPY found to consistently inhibit EAE (50 μg/kg) and 100 μg/kg of the Y_1 receptor antagonist BIBO3304. Interestingly, blocking Y_1 receptors with BIBO3304 abrogated the suppressive effect of NPY on EAE (Fig. 2*A*). This indicates that NPY probably inhibits clinical signs of EAE via Y_1 receptors. To further clarify this point, we treated the mice with a novel Y_1 receptor agonist, [D-His^{26}]NPY (Table I). Preliminary experiments showed that this compound is very potent and that a smaller dose (0.1 μg/kg to 0.01 μg/kg) than that for NPY effectively suppresses EAE. Due to a limited amount of the compound available, we treated the mice with 0.01 μg/kg of [D-His^{26}]NPY on alternate days until the end of the experiment. As shown in Fig. 2*B* and Table II, this Y_1 receptor agonist significantly down-regulated the clinical course of EAE, further supporting the role of Y_1 receptor in the NPY-mediated suppression of EAE. We also examined the effect of another Y_1 receptor agonist, [F^7,P^{34}]NPY, on EAE at 0.01, 0.1, and 1 μg/kg. Unlike [D-His^{26}]NPY, [F^7, P^{34}]NPY was not effective at 0.01 or 0.1 μg/kg. However, treatment with 1 μg/kg [F^7,P^{34}]NPY every other day significantly ameliorated clinical signs of EAE (Table II). In contrast, treatment with a selective Y_5 receptor agonist, [Ala^{31},Aib^{32}]NPY, did not show any effect on the clinical course of EAE (Table II). Taken together, these experiments strongly indicate that exogenous NPY suppresses the clinical signs of EAE through the activation of Y_1 receptors.

Next we asked whether endogenous NPY plays a role in the natural course of EAE. To answer this question, we evaluated the clinical course of EAE in mice treated with the Y_1 receptor antagonist BIBO3304. Blocking Y_1 receptors with BIBO3304 led to a significantly earlier onset of disease (Table II), although severity of EAE after onset was not significantly altered. This indicates that endogenous NPY prevents premature development of EAE by interfering with the induction of MOG_{35-55}-specific autoimmune T cells, but it is inefficient to modulate the effector phase of EAE.

Y_1 receptor agonist inhibits induction phase of EAE

We attempted to treat the mice with the Y_1 receptor agonist [D-His^{26}]NPY after appearance of the first clinical signs of EAE. However, the treatment protocols starting after onset of clinical manifestations did not significantly alter the clinical course of EAE (data not shown), indicating that Y_1 receptor stimulation could not modify the effector phase of EAE. In contrast, alternate day administration of the D-His^{26} compound during the induction phase of EAE (from day 0 to 10) after sensitization significantly inhibited the development of EAE (Fig. 3). In fact, the induction phase treatment (days 0–10) was as efficient as the long term treatment

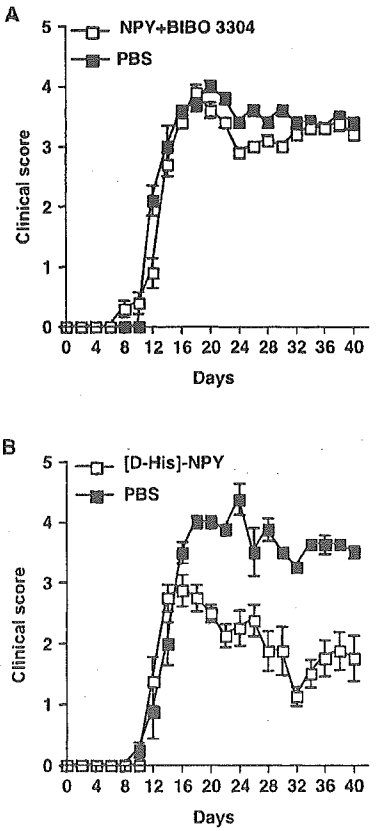

FIGURE 2. Receptor specificity of the suppressive effect of NPY. *A*, The suppressive effect of NPY is abrogated when NPY is administered in combination with the Y_1 receptor antagonist BIBO3304 (100 μg/kg). *B*, The novel and highly selective Y_1 receptor agonist [D-His^{26}]NPY (0.01 μg/kg) induced a similar suppression of EAE as seen with NPY (Fig. 1*A*). Data represent mean ± SEM. Additional disease parameters are analyzed in Table I.

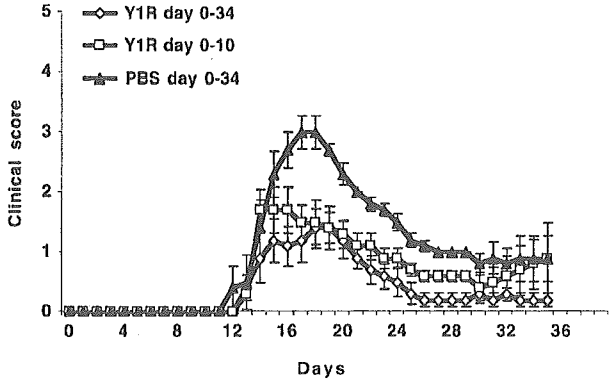

FIGURE 3. [D-His^{26}]NPY treatment from day 0 to day 10 significantly suppresses development of EAE. The B6 mice sensitized with MOG_{35-55} were treated i.p. with [D-His^{26}]NPY (0.01 μg/kg) every other day either throughout the experiment (Y1R day 0–34) or during the induction phase (Y1R day 0–10). Compared with control mice that were given PBS on alternate days, the mice treated with [D-His^{26}]NPY showed milder clinical course. The effect of the treatment from day 0 to day 10 is comparable with the continuous treatment from day 0 to 34. Data represent mean ± SEM. Each treatment group consists of five mice.

(days 0–34) covering both induction and effector phases. This result implies that Y_1 receptor stimulation leads to the inhibition of induction, but not effector phase of EAE.

EAE suppression is associated with an inhibition of MOG_{35-55}-specific Th1 response

NPY has been demonstrated to alter the cytokine profile of in vitro established Th1 clones toward Th2 directions (22, 23). We therefore speculated that the inhibitory action of NPY on EAE might be due to a modulation of the Th1/Th2 balance resulting from a Th2 bias of MOG_{35-55}-reactive T cells. To explore this possibility, we first measured serum levels of IgG1 and IgG2a isotypes of anti-MOG_{35-55} Abs at day 40 after immunization. It is generally accepted that elevation of Ag-specific IgG2a Ab results from the augmentation of a Th1 immune response to the Ag, whereas a higher level of IgG1 Ab reflects a stronger Th2 response to the Ag. Fig. 4*A* demonstrates that the treatment with NPY and the Y_1 receptor agonists remarkably inhibits anti-MOG_{35-55} IgG2a titers, but they do not significantly alter IgG1 titers. Consequently, the IgG1-IgG2a ratio was significantly elevated in mice treated with either NPY or the Y_1 receptor agonists, indicating that the suppression of EAE after NPY treatment is associated with a Th2 bias of MOG_{35-55}-reactive autoimmune T cells (Fig. 4*B*).

Treatment with the Y_1 receptor agonist inhibits the ex vivo production of IFN-γ by MOG_{35-55}-specific T cells

To further characterize the immunomodulatory properties of NPY in vivo, we isolated the draining LN cells at day 10 from mice treated with [D-His^{26}]NPY and from control mice treated with PBS and stimulated the lymphoid cells with MOG_{35-55} in vitro. We compared these two groups with respect to the levels of IFN-γ and IL-4 in the culture supernatant and cell-proliferative responses. We found that in vivo treatment with the Y_1 receptor agonist significantly inhibited the production of IFN-γ on in vitro stimulation with MOG_{35-55} (Fig. 5*A*). [D-His^{26}]NPY seemed to slightly inhibit the proliferation of MOG_{35-55}-specific T cells as well (Fig. 5*B*), but it was not statistically significant. IL-4 concentrations were below the detection level. These results indicate that the inhibition of IFN-γ production by MOG_{35-55}-specific T cells may underlie

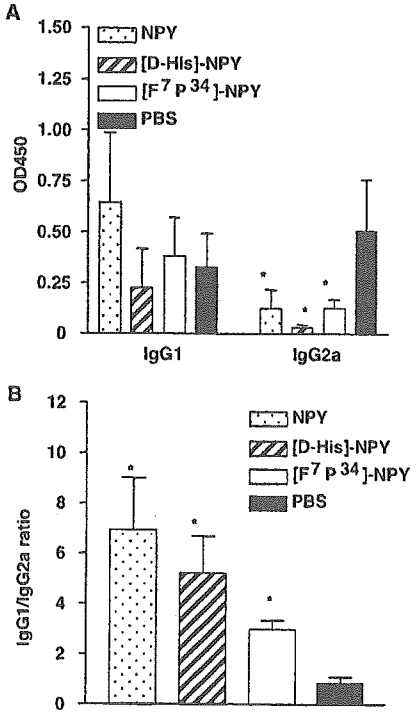

FIGURE 4. Analysis of anti-MOG_{35-55} Abs of IgG1 and IgG2a isotype after treatment with NPY and Y_1 receptor agonists. *A*, NPY (p = 0.0430) and the Y_1 receptor agonists [F^7,P^{34}]NPY (p = 0.0091) and [D-His^{26}]NPY (p = 0.0147) induced a significant inhibition of IgG2a. *B*, Assessment of the IgG1-IgG2a ratio revealed that NPY (p = 0.0309) and the Y_1 receptor agonists [F^7,P^{34}]NPY (p = 0.0054) and [D-His^{26}]NPY (p = 0.0417) would induce a Th2 bias of T cell response to MOG_{35-55}. Serum samples (n = 5 per condition) were obtained at day 40 after immunization and were analyzed as indicated in *Material and Methods*. Data represent mean ± SEM; *, significant differences between NPY/analogs and controls (PBS).

the Th2 deviation (a higher IgG1-IgG2a ratio) provoked by the Y_1 receptor agonist.

NPY treatment alters autoimmune T cells but not APC

To obtain insights into the altered Th1/Th2 balance through the activation of Y_1 receptors, we explored whether T cells or APCs are the major target of NPY. To address this question, we separated T cells from animals treated in vivo with either [D-His^{26}]NPY (treated) or PBS (untreated). The T cells were mixed with irradiated LN cells from treated or untreated mice, serving as APC, and then stimulated with 100 μg/ml MOG_{35-55} in vitro. Despite whether T cells from untreated mice (untreated T cells) were reconstituted with treated or untreated APC, they responded equally well to MOG_{35-55} with regard to the production of IFN-γ (Fig. 6*A*, Columns 3 and 4). However, when T cells from treated mice (treated T cells) were used for reconstitution (Fig. 6*A*, Columns 1 and 2), IFN-γ production was remarkably reduced regardless of the source of the APC (two ANOVA, p = 0.001). However, cell proliferation responses were not significantly different among the reconstituted populations (Fig. 6*B*). These results demonstrate that the in vivo effect of [D-His^{26}]NPY is mediated by the selective alteration of the T cell function to secrete IFN-γ but not of APC.

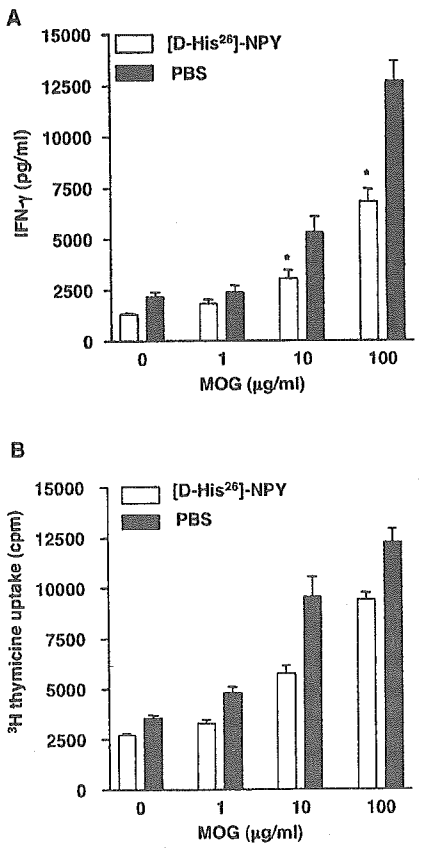

FIGURE 5. Comparison of MOG_{35-55}-specific T cell responses after in vivo treatment with [D-His^{26}]NPY and PBS. *A*, In vivo treatment with the Y_1 receptor agonist [D-His^{26}]NPY significantly inhibits the ability of MOG_{35-55}-specific T cells to secrete IFN-γ on Ag challenge (treatment vs control; 100 μg MOG_{35-55}, $p < 0.0001$). *B*, Tendency toward inhibition of the proliferative response of MOG_{35-55}-specific T cells. Statistical analysis revealed no significant differences (ANOVA). Popliteal and inguinal LN cells from treated and control animals were incubated in the presence of MOG_{35-55} for 48 h. IFN-γ was detected by ELISA, and proliferation was determined by estimating the uptake of [^{3}H]thymidine. Pooled data from three independent experiments is shown ($n = 9$). Error bars, SEM; *, significant differences.

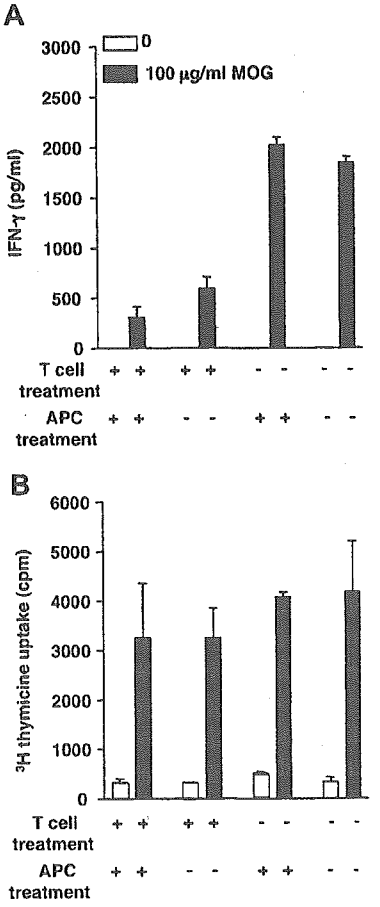

FIGURE 6. Effect of [D-His^{26}]NPY on primed T cells and APC. T cells and APC were isolated from animals treated with either [D-His^{26}]NPY in vivo (treated) or PBS (untreated) and then coincubated in the presence of MOG_{35-55} in vitro. *A*, Significant inhibition of the IFN-γ secretion is observed only in T cells from treated animals (two-way ANOVA: treated T cells vs untreated T cells, $p = 0.001$). Coincubation with either treated or untreated APC revealed no statistically significant differences. *B*, The proliferative response is not significantly different, although there is a tendency toward a decreased proliferation on treatment. Pooled data from two independent experiments is shown ($n = 6$). Error bars represent SEM.

Mouse T cells express Y_1 receptor mRNA and respond to the Y_1 receptor agonist in vitro

The in vivo results presented above strongly suggest that NPY acts on EAE via direct activation of Y_1 receptors expressed on the MOG_{35-55}-specific autoimmune T cells. To verify this further, we examined whether mouse T cells express the Y_1 receptor. As shown in Fig. 7, RT-PCR enabled us to detect the expression of Y_1 receptor mRNA in MOG_{35-55}-sensitized LN cells (Fig. 7*A*) and in spleen T cells isolated from naive mice (Fig. 7*B*). We also examined expression levels of the Y_1 receptor in T cells polarized in vitro toward Th1 or Th2, according to the described method (28). We saw no significant difference between Th1 and Th2 cells regarding the Y_1 receptor expression.

To further determine the functional significance of these findings, we stimulated spleen T cells with plate-bound anti-CD3 mAb in the presence of different concentrations of [D-His^{26}]NPY (10^{-12}–10^{-8} M), and measured IFN-γ in the supernatant. The results indicate that the Y_1 receptor agonist significantly inhibits the secretion of IFN-γ on stimulation with anti-CD3 Ab in a dose-dependent manner (Fig. 8). These data further support that the Th2 bias found in vivo is mediated through the activation of functional Y_1 receptors expressed on autoimmune T cells.

Effect of the Y_1 receptor agonist on EAE induced in SJL/J mice

Furthermore, we asked whether the Y1 receptor agonist might also modulate acute EAE actively induced with $PLP_{139-151}$ in SJL/J mice. We found that the continuous treatment from day 0 to day 30 significantly suppressed clinical EAE, regarding the maximum clinical score: D-His^{26}-treated mice, 1.8 ± 0.52 vs PBS-treated mice, 3.2 ± 0.37 ($p < 0.02$). Administration of D-His^{26} during the induction phase (from day 0 to day 10) also reduced the clinical severity of EAE as compared with treatment with PBS. It was interesting to know whether the treatment during the induction phase may inhibit the generation of encephalitogenic T cells reactive to $PLP_{139-151}$. To answer this question, we isolated $PLP_{139-151}$-sensitized lymphoid cells from D-His^{26}- or PBS-treated mice at day 10 after immunization and stimulated the cells in vitro with $PLP_{139-151}$. The activated T cells were adoptively transferred to naive SJL/J mice to induce passive EAE as described in *Materials and Methods*. Our protocol induced very serious EAE in the recipients ($n = 5$ for each

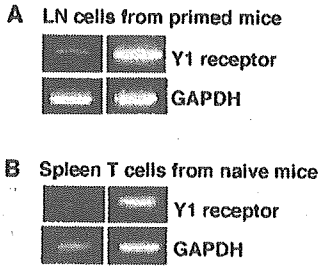

FIGURE 7. Constitutive expression of Y_1 receptor mRNA in LN cells from immunized animals and T cells from naive mice. *A*, LN cells from animals immunized with MOG_{35-55} express Y_1 receptor RNA (*left panels*). LN cells were prepared as described in *Material and Methods*. RNA from homogenized mouse brain was used as a positive control (*right panels* in both *A* and *B*). One representative experiment is shown. *B*, T cells isolated from spleens of naive animals also express Y_1 receptor RNA (*left panels*). Five micrograms of total RNA were used for RT-PCR, and amplified products were analyzed by agarose gel electrophoresis. GAPDH was used as a control for equal loading. The sizes of the PCR products were 334 bp for the Y_1 receptor and 190 bp for GAPDH.

group), and all the recipient mice died before day 18 after cell transfer. However, there was a clear tendency that mice transferred with T cell blasts from D-His^{26}-treated mice would survive for a longer period of time (the date of death in an individual mouse: day 14, day 17, day 17, day 17, day 17) compared with those given the T cell blasts from PBS-treated mice (the date of death: day 8, day 11, day 14, day 15, day 15). In addition, the mice transferred with the T cells from [D-His^{26}-treated mice showed a reduced clinical score at day 8 compared with the control mice (1.5 ± 0.5 vs 4.5 ± 0.6). These results indicate that NPY Y_1 agonist is effective also for EAE induced in SJL/J mice and that the mechanism of action is to interfere with the process of effector lymphocyte generation.

Discussion

With regard to the bidirectional interaction between the immune and the nervous system, prior studies have indicated that catecholamines released from sympathetic nerve endings regulate Th1 responses and Th1-mediated disease such as EAE. Our study demonstrates that NPY also plays an important role in the regulation of EAE. It has previously been indicated that NPY modulates various T cell functions in vitro, including T cell adhesion to integrins (30) and cytokine secretion of in vitro established T clones (22, 23). Our study could be regarded as the first work to provide evidence that NPY modulates T cell function in vivo and that NPY is involved in the natural regulation of Th1-mediated autoimmunity.

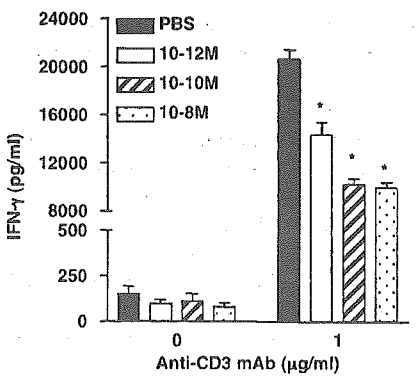

FIGURE 8. Splenocytes from naive mice were stimulated by a plate-bound anti-CD3 mAb in the presence of various concentrations of [D-His^{26}]NPY. The Y_1 receptor agonist induced a dose-dependent inhibition of the secretion of IFN-γ in vitro (Y_1 receptor agonist vs control: 10^{-12} M, $p = 0.023$; 10^{-10} M, $p = 0.001$; 10^{-8} M, $p < 0.0001$). Pooled data from three independent experiments are shown ($n = 7$). Error bars, SEM; *, significant differences between treated and untreated conditions.

The action of NPY is mediated via distinct receptor subtypes such as the Y_1, Y_2, Y_4, and Y_5 receptor. Here we conclude that Y_1 receptors are the main receptor subtypes engaged in the NPY-mediated suppression of EAE. This conclusion was obtained from a series of experiments applying Y_1 receptor agonists and a Y_1 receptor antagonist. Firstly, we showed that the suppressive effect of NPY on EAE was abolished when we coinjected the Y_1 receptor antagonist BIBO3304. Secondly, we replaced native NPY with two types of compounds known to selectively stimulate Y_1 receptors ([D-His^{26}]NPY, [F^7,P^{34}]NPY) and found that these NPY analogs also effectively suppress the development of EAE. However, a Y_5 receptor agonist was not effective. Thirdly, treatment with BIBO3304 resulted in a significantly earlier onset of disease. All of these results indicate that Y_1 receptor engagement leads to the suppression of EAE.

The Y_1 receptor agonists appear to be much more potent EAE inhibitors than native NPY. In terms of the dosage requirement to gain clinical effects, the hierarchy for the native and altered NPY compounds was apparent ([D-His^{26}]NPY > [F^7,P^{34}]NPY > native NPY). It seems that the efficacy of these ligands as EAE therapeutics correlates with the specificity for the Y_1 receptors. Namely, [D-His^{26}]NPY is more selective for Y_1 receptors, compared with [F^7,P^{34}]NPY (24). Taking this into consideration, it is possible that stimulation of non-Y_1 receptors may compete with Y_1 receptor ligation. Alternatively, the Y_1 receptor agonists may be more efficacious as ligands than native NPY in inducing intracellular events leading to the immunoregulation. Alternatively, these differences in the potency of Y_1 receptor compounds and the native peptide NPY may be explained by parallel activation of stimulatory and inhibitory Y receptors by NPY itself rather than by the specific ligands. Furthermore, it is possible that the different Y_1 receptor specific compounds exhibit a differential tissue penetration or may be differentially degraded by specific enzymes such as CD26 (31, 32). Further studies are needed to verify these postulates and provide us with a new insight into NPY-Y_1 receptor interactions.

The experiment using the Y_1 receptor antagonist BIBO3304 showed an earlier onset of EAE, although the disease course was not altered after onset. This indicates that endogenous NPY plays a regulatory role in the induction phase, but not in the effector phase of EAE. Consistent with this, we showed that the treatment during the induction phase is as effective as the continuous treatment covering both induction and effector phases, whereas the treatment starting after onset of EAE does not change the clinical course of EAE. A possible explanation for the failure of the Y_1 receptor antagonist to alter the effector phase is that endogenous NPY levels may substantially decrease in the effector phase, owing to enzymatic degradation. Whereas it is currently impossible to measure the NPY levels in mice with EAE, it is well known that NPY is cleaved by enzymes such as dipeptidyl peptidase IV (CD26), a membrane-bound enzyme, constitutively expressed on numerous cells including leukocytes (31, 32). In addition, activated T cells are reported to express a higher level of CD26 on their surface (33). Thus, it is possible that the contribution of endogenous NPY to the immunoregulation might be reduced in the effector phase because of the rapid degradation by enzymes such as CD26. It is necessary to pursue this issue with different methodologies.

Regarding the mechanism for NPY-mediated suppression of EAE, we showed a significant inhibition of anti-MOG_{35-55} IgG2a titers and of IFN-γ production by MOG_{35-55}-specific T cells after treatment with [D-His^{26}]NPY. In contrast, IgG1 titers were unaffected, which resulted in a higher IgG1-IgG2a ratio in treated mice. Also, T cell-proliferative responses were not affected significantly. On the basis of these results, we conclude that NPY plays a selective role in inhibiting IFN-γ production by MOG_{35-55}-specific T cells, leading to a Th2 bias. Ex vivo reconstitution experiments also showed that MOG_{35-55}-specific T cells are the major target for NPY. Although the presence of functional Y_1 receptors on rat leukocytes has been documented (20, 21), we proved here the presence of mRNA encoding the Y_1 receptor in T cell populations. Accompanying in vitro experiments further confirmed that NPY suppresses T cell production of IFN-γ provoked by CD3 cross-linking.

In conclusion, this study demonstrates for the first time to our knowledge that NPY has an immunomodulatory activity that suppresses signs of EAE. Given that the levels of NPY in the CSF are reduced in patients with MS (34, 35), it is tempting to speculate that NPY may also play a critical role in preventing the development of MS. With the availability of novel and highly selective agonists and their ability to mimic the effects of NPY in a highly specific manner, we propose that targeting NPY receptors may be a promising new therapeutic approach to autoimmune disorders.

Acknowledgments

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Suppression of Collagen-Induced Arthritis by Natural Killer T Cell Activation With OCH, a Sphingosine-Truncated Analog of α-Galactosylceramide

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Objective. **OCH, a synthetic analog of α-galactosylceramide with a truncated sphingosine chain, stimulates natural killer T (NKT) cells to produce predominantly Th2 cytokines. Thus, OCH may be a potential agent for the treatment of Th1-mediated autoimmune diseases. This study was designed to evaluate the protective effects of OCH on collagen-induced arthritis (CIA) in mice.**

Methods. **Mice were immunized with type II collagen (CII) and injected intraperitoneally twice per week with OCH, before or after the onset of CIA. They were monitored to assess the effect of OCH treatment on the severity of disease. Anti-CII antibodies and cytokine production were measured by enzyme-linked immunosorbent assay. Expression of cytokine genes was determined by quantitative reverse transcriptase–polymerase chain reaction.**

Results. **OCH inhibited CIA in wild-type C57BL/6 (B6) mice but not in NKT-deficient mice. OCH suppressed CIA in SJL mice, which are prone to autoimmune diseases and have a deficiency in the number and function of NKT cells which is similar to that in patients with autoimmune diseases, even after disease has already developed. Disease protection conferred by OCH correlated with its ability to selectively induce Th2 cytokine production mediated by NKT cells and to promote collagen-specific Th2 responses. Neutralization of interleukin-4 (IL-4) or IL-10 with monoclonal antibodies abolished disease protection by OCH, indicating a critical role for these cytokines.**

Conclusion. **Taken together, our findings suggest that OCH holds possibilities as a therapeutic agent for autoimmune diseases such as rheumatoid arthritis.**

Rheumatoid arthritis (RA) is a common autoimmune disease characterized by persistent inflammation of joints resulting in progressive destruction of cartilage and bone. Although its precise etiology is not clearly understood, cumulative evidence suggests that Th1 cells secreting interferon-γ (IFNγ) and tumor necrosis factor α (TNFα) exacerbate disease, whereas Th2 cells producing interleukin-4 (IL-4) and IL-10 suppress arthritis (1). Studies with animal models have demonstrated that systemic or locally administered IL-4 and IL-10 can effectively protect against arthritis in mice (2–11).

Natural killer T (NKT) cells are a unique subset of T cells that coexpress T cell receptor α/β (TCRα/β) and receptors from the NK lineage. NKT cells express an invariant TCRα chain (encoded by a $V_\alpha14$–$J_\alpha281$ rearrangement in mice and a homologous $V_\alpha24$–$J_\alpha Q$ rearrangement in humans). Unlike conventional T cells that recognize peptides in association with major histocompatibility complex (MHC), NKT cells recognize glycolipid antigens bound to the nonpolymorphic class I MHC–like protein, CD1d. NKT cells have been implicated in a variety of immune responses such as infection and tumor immunity. One striking feature of NKT cells is their capacity to secrete a large amount of cytokines,

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including IL-4 and IFNγ, in response to TCR stimulation (12–14). Recently, a number of reports have indicated that NKT cells play a critical role in the regulation of autoimmune responses. Abnormalities in the numbers and functions of NKT cells have been observed in patients with autoimmune diseases (15–18) as well as in a variety of mouse strains that are genetically predisposed to the development of autoimmune diseases (19–23).

While the natural ligand for NKT cells remains to be determined, α-galactosylceramide (α-GC) (Figure 1A), a derivative of marine sponge, has been shown to bind to CD1d and strongly stimulate NKT cells to produce IFNγ and IL-4, both in humans and in mice (24–26). Previously, we have shown that OCH (Figure 1A), an analog of α-GC with a truncated sphingosine chain, efficiently inhibits induced experimental autoimmune encephalomyelitis (EAE) in C57BL/6 (B6) mice, due to its ability to stimulate NKT cells to selectively produce Th2 cytokines; in contrast, α-GC had little effect on EAE (27,28).

In the present study, we found that OCH inhibits collagen-induced arthritis (CIA), a murine experimental model for RA, in wild-type B6 but not NKT-deficient J_α281-knockout mice. We also demonstrated that OCH inhibits CIA in SJL mice even after arthritis has already developed. Experiments with anti–IL-4 or anti–IL-10 administration revealed that IL-4 and IL-10 are critical for OCH-mediated suppression of CIA. These results suggest that stimulation of NKT cells with OCH could be an attractive means of intervention in autoimmune diseases such as RA.

MATERIALS AND METHODS

Mice. B6 mice were purchased from Clea Laboratory Animal Corp. (Tokyo, Japan). SJL mice were obtained from Charles River Japan (Yokohama, Japan). J_α281-knockout mice were kindly provided by Dr. Masaru Taniguchi (Chiba University Graduate School of Medicine, Chiba, Japan). The animals were kept under specific pathogen-free conditions and studied at 7–10 weeks of age.

Induction of CIA. Mice were immunized intradermally at the base of the tail with 100 μg of either chicken type II collagen (CII) (for B6 mice) or bovine CII (for SJL mice) (Collagen Research Center, Tokyo, Japan) emulsified with an equal volume of Freund's complete adjuvant (CFA), containing 250 μg of H37RA *Mycobacterium tuberculosis* (Difco, Detroit, MI). The animals were boosted by intradermal injection with the same antigen preparation on day 21. Mice were examined for signs of joint inflammation 3 times per week, and joint involvement was scored as follows: 0 = no change, 1 = focal redness of the limb or swelling and redness of 1 digit, 2 = mild swelling and erythema of the limb or swelling of >2 digits, 3 = marked swelling and erythema of the limb, 4 = maximal

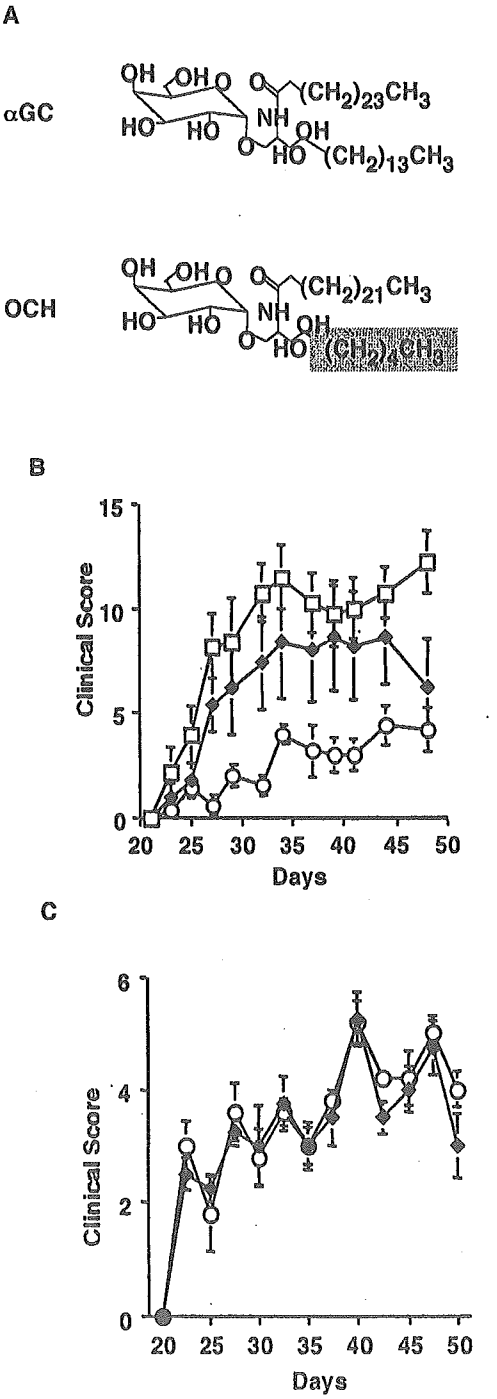

Figure 1. A, Structure of α-galactosylceramide (α-GC) and OCH, a sphingosine-truncated analog of α-GC. B and C, Effect of OCH on collagen-induced arthritis (CIA) in C57BL/6 (B6) and J_α281-knockout mice. B, Clinical score of CIA in B6 mice treated with 500 μg/kg of α-GC (◆), OCH (○), or vehicle (□) twice per week starting from day 21. C, Clinical score of CIA in J_α281-knockout mice treated with 500 μg/kg of OCH (○) or vehicle (◆) twice per week starting from day 21. Data shown are from a single experiment representative of 2 identical experiments; values are the mean ± SEM (5 mice per group).