ƒ ‹ F | Œfia‹ ˙'e›ß• |–M^D‹ ª¤s›p e ·`⁄⁄¶i« ‡ł§i

„L« ¿}⁄˘‡ † †£"«„ fl fiqfl Tfi§¶ ˙»…"˝…v¯T(2/3)

›p e^ §¡G §O«‹ ›p e

›p e‰ ¸s„¡GNSC92-2314-B-038-039-

ıƒ ·`¶¡¡G92 ƒ~08 ⁄°01 ⁄ Ø 93 ƒ~07 ⁄°31 ⁄Ø

ıƒ ‡ ƒ ¡G» O ¥´ ˙⁄j ˙´ ¤ § ‡N¤t

›p e¥ D ø⁄¡HG§ ı §´

‡ł§i^ «‹¡G" °†‡ł§i

‡ B † zŁ¡fG¡¥»›p e¥ i ⁄ ‰ ¶‹ d,

⁄⁄ ¥` Œ 93 ƒ~5 ⁄°26 ⁄Ø

ƒ ‹ F | Œfia‹ ·'e›ß• |, §U–M^D‹ ª¤s›p eƒ¤"G‡ł§i

¡ ¡ ¡

Abstract

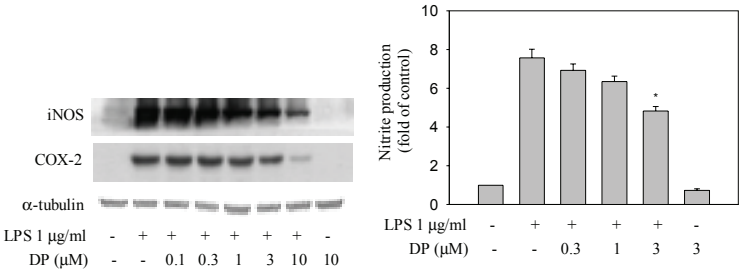
Dipyridamole is a nucleoside transport inhibitor and a non-selective phosphodiesterase inhibitor that increases intracellular level of cAMP and cGMP through phosphodiesterase inhibition. Type 4 phosphodiesterase has been demonstrated to have anti-inflammatory effects in many experimental systems. This study investigates whether dipyridamole inhibits lipopolysaccharide (LPS)-induced inducible nitric oxide (iNOS) and cyclooxygenase (COX-2) expression in RAW 264.7 macrophages. Treatment of RAW 264.7 macrophages with LPS caused dose- and time-dependent increases in iNOS and COX-2 expression. Treatment of cells with dipyridamole blocked the LPS-induced iNOS and COX-2 expression. Dipyridamole inhibited NF-κB activation as demonstrated by inhibition of IκB phosphorylation, IκB degradation, p65 NF-κB transloction and the transcription of reporter gene. Dipyridamole also inhibited LPS-stimulated p38 MAPK and IKK-β activities in RAW 264.7 cells. A p38 mitogen-activated protein kinase (MAPK) inhibitor, SB203580, inhibited LPS-stimulated iNOS expression and IKK-β activation suggesting LPS may activate NF-κB signaling pathway via upstream p38 MAPK activation. Furthermore, dipyridamole stimulated a transient activation of mitogen-activated protein kinase phosphatase 1 (MKP-1), a potent inhibitor of p38 MAPK. Taken together, these data suggest that dipyridamole exerts anti-inflammatory effect via activation of MKP-1, which dephosphorylates and inactivates p38 MAPK. Inactivation of p38 MAPK in turn inhibits IKK-β activation and subsequent NF-κB signaling pathway that mediates LPS-induced iNOS and COX-2 expression in RAW 264.7 cells. These results support the notion that dipyridamole may have anti-inflammatory effects.

Keywords: Dipyridamole, NOS, LPS, NF-kappa B, RAW 264.7 macrophages.

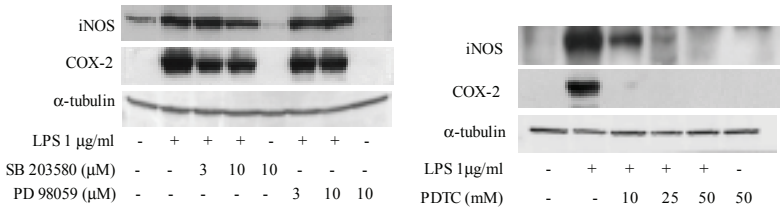
Kidney International (65) 1664-1675

⁄T¡B †"G»P Q‰

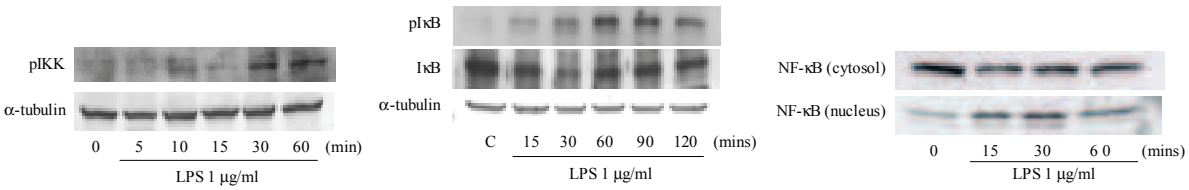
3.1. Dipyridamole inhibits LPS-induced iNOS and COX-2 expression and nitrite accumulation in RAW 264.7 cells.



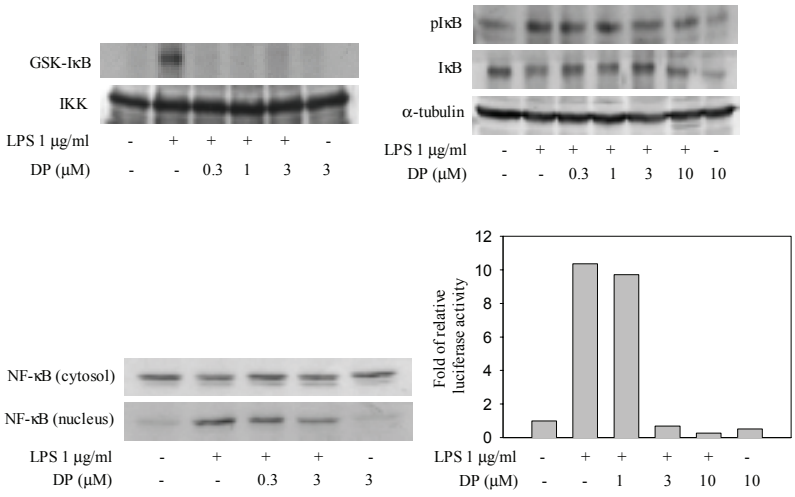
3.2. LPS-induced iNOS and COX-2 expression is mediated through p38MAPK and NFkB pathways.



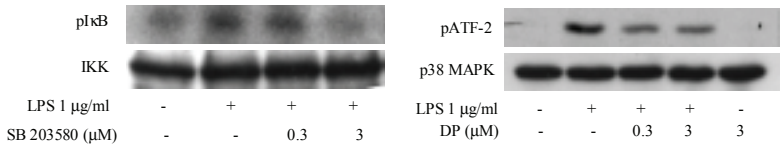
3.3. Effect of LPS on IKK phosphorylation, IkB phosphorylation, IkB degradation and NF-κB translocation in RAW 264.7 cells.



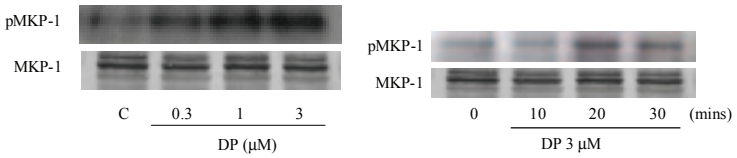
3.4. Dipyridamole inhibits LPS-induced IKK activity, IkB phosphorylation, IkB degradation, NF-κB translocation and NF-κB responsive luciferase reporter gene expression in RAW 264.7 cells.



3.6. Effect of SB203580 on LPS-stimulated on IKK-β activity in RAW 264.7 cells. Dipyridamole inhibits LPS-activated p38 MAPK in RAW 264.7 cells.



3.7. Dipyridamole increases MKP-1 phosphorylation in RAW 264.7 cells.



¡G

Dipyridamole is a non-selective phosphodiesterase inhibitor, which has been shown to improve proteinurea in membranous glomerulonephritis, mesangial IgA glomerulonephritis, and segmentary and focal hyalinosis glomerulonephritis (Harmankaya et al., 2001). Inhibition of proteinuria in glomerulonephritis has been attributed to platelet response (Camara et al., 1991). However, there is no consistent evidence supporting dipyridamole as an effective antithrombotic agent in cardiovascular and renal diseases. Because many PDE inhibitors suppress the LPS-stimulated cytokine production (Yoshigawa et al., 1999), we have sought to resolve the question of whether dipyridamole exerts an anti-inflammatory effect. LPS is a bacterial endotoxin, which induces the expression of a number of proteins associated with inflammation. LPS-induced iNOS and COX-2 expression are key mediators in inflammatory responses. In the present study, we investigate whether dipyridamole has an effect on LPS-induced iNOS and COX-2 expression. We present evidences showing that dipyridamole inhibits iNOS and COX-2 expression in LPS-stimulated RAW 264.7 cells. Because inhibition of p38 MAPK by a pharmacological specific inhibitor, SB203580, is enough to suppress the IKK-β activity, these data suggest that dipyridamole inhibits p38 MAPK activation leading to an inhibition of IKK-β and the NF-κB signaling pathway, and subsequently suppresses LPS-induced iNOS and COX-2 expression in RAW 264.7 macrophages. In addition to these novel findings, we demonstrated that dipyridamole stimulates MKP-1 activation, which lead to p38 MAPK and IKK-β inactivation and NF-κB specific transcription.

Our data agree with many other reports showing phosphodiesterase inhibitors are immunoregulators and can be used as an anti-inflammatory agent (Bielekova et al., 2000; Burnouf et al., 2002). Dipyridamole exerts beneficial effects on glomerulonephritis (Camara etal., 1991) and blocks the lipopolysaccharide (LPS)-induced increase in monocyte-associated tissue factor activity (Brozna et al., 1990). LPS-activation of p38MAPK and NF-kB signal transduction pathways may contribute to inflammatory responses and disease progression. In

this study, we demonstrated that dipyridamole activates MKP-1, which in turn inactivates these proinflammatory signaling pathways. Our results support the notion, that dipyridamole can serve as a anti-inflammatory agent as well.

The results of our inhibitor studies suggest that the LPS-induced iNOS and COX-2 expressions is a consequence of the activation of p38 MAPK, IKK, and NF-κB in RAW 264.7 cells. Activation of the transcription factor, NF-κB, is responsible for the altered transcription of iNOS and COX-2 in macrophages and many other cell types (Huttunen et al., 1999; Mohamed et al., 1999; Li et al., 2000). Consistently, we have found that NF-κB activation plays an important role in LPS-induced iNOS expression. This notion is supported by the data herein: *1)* Pretreatment of cells with PDTC inhibited LPS-mediated iNOS induction; *2)* Treatment of RAW cells with LPS increased p65 NF-κB translocation from the cytosol to the nucleus; *3)* LPS treatment phosphorylated and degraded IκB in the cytosol; and 4) Treatment of RAW cells with LPS enhanced NF-κB-specific transcription as demonstrated by the expression of reporter gene, luciferase activity.

NF-κB can be activated in response to a broad range of stimuli and conditions, including interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) (Bowie and O'Neill, 2000). LPS triggers a signaling pathway resulting in the production of inflammatory cytokines, which include TNF α and IL-1. Thus, LPS activation of NF-κB may well be an indirect effect due to the release of cytokines. Indeed, LPS induces TNF α secretion through nuclear factor kappa B in human vascular muscle cells.

The intracellular signaling mechanisms by which LPS induce NF-κB remain to be determined. LPS may activate protein tyrosine kinase, p21 Ras, protein kinase C-βII (PKC βII), or p42/44MAPK. In agreement, we previously found that protein tyrosine kinase, p21 Ras, and p38 MAPK are involved in LPS-induced iNOS expression in A549 cells (Lin et al., 2001). LPS stimulation of human monocytes activates several intracellular signaling pathways that include the p38 mitogen-activated protein kinase (MAPK) pathway (Guha and Mackman, 2001). Lipoteichoic acid activation of NF-κB is mediated through protein tyrosine kinase (Kengatharan al., 1996). Activation of NF-κB in human monocytes involves PKC and PI-3K (D'Addario et al., 1999; Diaz-Guerra et al., 1999). Thus, LPS may activate tyrosine kinase, PI-3K, PKC, and p38 MAPK, which in turn activate IκB kinase (IKK), resulting in NF-kappaB (p50/p65) translocation and the induction of many genes encoding inflammatory mediators including iNOS. However, given the addition of MEK-specific inhibitor, PD 98059, fails to normalize the LPS-induced iNOS expression, and the facts that many other pathways could contribute to the activation of NF-κB, which may play a role in the NF-κB dependent induction of iNOS expression. It is possible that other signaling pathways other than those highlighted in the present study may also contribute to the activation of LPS in RAW264.7 cells.

In conclusion, our results suggest that dipyridamole may have anti-inflammatory effects. Our results clearly demonstrated that dipyridamole inhibits LPS-induced inflammatory

mediator expression in RAW 264.7 macrophages through p38 MAPK, IKK, and NF-κB dependent mechanisms. Whether dipyridamole exerts these effects in clinical treatment warrant further investigation.

Baeuerle, P.A., and Baltimore, D., 1996. NF-κB:,Ten years after. Cell 87,13-20.

Baldwin, J.A.S., 1996. The NF-κB and IκB proteins: new discoveries and insights. Annu. Rev. Immunol. 14, 649-681.

Bielekova B. Lincoln A. McFarland H. Martin R. 2000, Therapeutic potential of phosphodiesterase-4 and -3 inhibitors in Th1-mediated autoimmune diseases. J Immunol 164(2):1117-24,

Bowie, A., and O'Neill, L.A.J., 2000. Oxidative stress and nuclear factor –kB activation. Biochem. Pharmacol. 59, 13-23.

Brozna JP. Horan M. Carson SD. , 1990. Dipyridamole inhibits O2- release and expression of tissue factor activity by peripheral blood monocytes stimulated with lipopolysaccharide. Thrombosis Research. 60(2):141-56.

Brown, K., Gerstberger, S., Carlson, L., Franzoso, G., and Siebenlist, U., 1995. Control of Iκ-B proteolysis by site specific signal induced phosphorylation. Science 267, 1485-1488.

Burnouf C. Pruniaux MP. , 2002. Recent advances in PDE4 inhibitors as immunoregulators and anti-inflammatory drugs. Current Pharmaceutical Design. 8(14):1255-96

Camara S. de la Cruz JP. Frutos MA. Sanchez P. Lopez de Novales E. Sanchez E. Sanchez de la Cuesta F. , 1991 Effects of dipyridamole on the short-term evolution of glomerulonephritis. Nephron. 58(1):13-6

Chen, Z., 1995. Signal-induced site-specific phosphorylation targets IκBα to the ubiquitin-proteasome pathway. Gene. Dev. 9, 1586-1597.

Chen P. Li J. Barnes J. Kokkonen GC. Lee JC. Liu Y. , 2002 Restraint of proinflammatory cytokine biosynthesis by mitogen-activated protein kinase phosphatase-1 in lipopolysaccharide-stimulated macrophages. J Immunol 169(11):6408-16

Clark AR. , 2003 MAP kinase phosphatase 1: a novel mediator of biological effects of glucocorticoids? J. Endocrinol. 178(1):5-12

D'Addario, M., Ahmad, A., Xu, J.W., Menezes, J., 1999. Epstein-Barr virus envelope glycoprotein gp350 induces NF-kappaB activation and IL-1beta synthesis in human monocytes-macrophages involving PKC and PI3-K. FASEB J. 13, 2203-2213.

Diaz-Guerra, M.J., Castrillo, A., Martin-Sanz, P., Bosca, L., 1999. Negative regulation by phosphatidylinositol 3-kinase of inducible nitric oxide synthase expression in macrophages. J. Immunol. 162, 6184-6190.

Engelbrecht Y. de Wet H. Horsch K. Langeveldt CR. Hough FS. Hulley PA. , 2003 Glucocorticoids induce rapid up-regulation of mitogen-activated protein kinase phosphatase-1 and dephosphorylation of extracellular signal-regulated kinase and impair proliferation in human and mouse osteoblast cell lines. Endocrinol 144(2):412-22

Gibbs CR. Lip GY. *, 1998* Do we still need dipyridamole? *Brit J Clin Pharmacol. 45(4):323-8*

Guha, M., Mackman, N., 2001. LPS induction of gene expression in human monocytes. Cellular Signalling 13, 85-94.

Haddad JJ. Land SC. Tarnow-Mordi WO. Zembala M. Kowalczyk D. Lauterbach R. , 2002 Immunopharmacological potential of selective phosphodiesterase inhibition. II. Evidence for the involvement of an inhibitory-kappaB/nuclear factor-kappaB-sensitive pathway in alveolar epithelial cells. J Pharmacol Exp Therap 300(2):567-76

Harmankaya O. Baturk T. Ozturk Y. Karabiber N. Obek A. , 2001.Effect of acetylsalicylic acid and dipyridamole in primary membranoproliferative glomerulonephritis type I. International Urology & Nephrology. 33(3):583-7

Hatzelmann A. Schudt C. Anti-inflammatory and immunomodulatory potential of the novel PDE4 inhibitor roflumilast in vitro. J Pharmacol Exp Therap 297(1):267-79, 2001

Hewitson TD. Tait MG. Kelynack KJ. Martic M. Becker GJ. , 2002 Dipyridamole inhibits in vitro renal fibroblast proliferation and collagen synthesis. J Lab Clin Med 140:199-208

Hori, O., Yan, S.D., Ogawa, S., Kuwabara, K., Matsumoto, M., Stern, D., and Schmidt, A. M., 1996. The receptor for advanced glycation end-products has a central role in mediating the effects of advanced glycation end-products on the development of vascular disease in diabetes mellitus. Nephrol. Dial. Transplant. 11, 13-16.

Hung KY. Shyu RS. Fang CC. Tsai CC. Lee PH. Tsai TJ. Hsieh BS.Dipyridamole inhibits human peritoneal mesothelial cell proliferation in vitro and attenuates rat peritoneal

fibrosis in vivo. Kidney International. 59(6):2316-24, 2001

Huttunen, H. J., Fages, C., and Rauvala, H., 1999. Receptor for advanced glycation end products (RAGE)-mediated neurite outgrowth and activation of NF-kappaB require the cytoplasmic domain of the receptor but different downstream signaling pathways. J. Biol. Chem. 274, 19919-19924.

Ichiyama T. Hasegawa S. Matsubara T. Hayashi T. Furukawa S., 2001 Theophylline inhibits NF-kappa B activation and I kappa B alpha degradation in human pulmonary epithelial cells. Naunyn-Schmiedebergs Archives of Pharmacology. 364(6):558-61

Imasato A. Desbois-Mouthon C. Han J. Kai H. Cato AC. Akira S. Li JD. , 2002 Inhibition of p38 MAPK by glucocorticoids via induction of MAPK phosphatase-1 enhances nontypeable Haemophilus influenzae-induced expression of toll-like receptor 2. J Biol Chem . 277(49):47444-50

Jacob A. Molkentin JD. Smolenski A. Lohmann SM. Begum N. , 2002 Insulin inhibits PDGF-directed VSMC migration via NO/ cGMP increase of MKP-1 and its inactivation of MAPKs. Am J Physiol - Cell Physiol. 283(3):C704-13

Kassel O. Sancono A. Kratzschmar J. Kreft B. Stassen M. Cato AC. Glucocorticoids inhibit MAP kinase via increased expression and decreased degradation of MKP-1. EMBO J 20(24):7108-16, 2001

Kengatharan, M., De Kimpe, S.J., Thiemermann, C., 1996. Analysis of the signal transduction in the induction of nitric oxide synthase by lipoteichoic acid in macrophages. Bri. J. Pharmacol. 117, 1163-1170.

Khechai, F., Ollivier,V., Bridey, F., Amar, M., Hakim, J., and de Prost, D., 1997. Effect of advanced glycation end product-modified albumin on tissue factor expression by monocytes. Role of oxidant stress and protein tyrosine kinase activation. Arterioscler. Thromb. Vasc. Biol., 17, 2885-2890.

Lander, H.M., Tauras, J.M., Ogiste, J.S., Hori, O., Moss, R.A., and Schmidt, A.M., 1997. Activation of the receptor for advanced glycation end products triggers a p21(ras)-dependent mitogen-activated protein kinase pathway regulated by oxidant stress. J. Biol. Chem. 272, 17810-17814.

Lasa M. Abraham SM. Boucheron C. Saklatvala J. Clark AR. Dexamethasone causes sustained expression of mitogen-activated protein kinase (MAPK) phosphatase 1 and phosphatase-mediated inhibition of MAPK p38. Molecular & Cellular Biology. 22(22):7802-11, 2002

Lenz TL. Hilleman DE.Aggrenox: a fixed-dose combination of aspirin and dipyridamole. Annals of Pharmacotherapy. 34(11):1283-90, 2000.

Li, Y.M., Mitsuhashi, T., Wojciechowicz, D., Shimizu, N., Li, J., Stitt, A., He, C., Banerjee,. D., Vlassara, H., 1996. Molecular identity and cellular distribution of advanced glycation endproduct receptors: relationship of p60 to OST-48 and p90 to 80K-H membrane

proteins. Proc. Nal. Acad. Sci. (USA). 93, 11047-11052.

Li, Y.H., Yan, Z.Q., Jensen, J.S., Tullus, K., Brauner, A., 2000. Activation of nuclear factor kappaB and induction of inducible nitric oxide synthase by Ureaplasma urealyticum in macrophages. Infect. Immun. 68, 7087-7093.

Lin, C.H., Lin, Y.F., Chang, M.C., Wu, C.H., Ho, Y.S., Lee, H.M., 2001. Advanced glycosylation end products induced iNOS expression in C6 glioma cells: Involvement of a p38 MAP kinase-dependent mechanism. Life Sci. 69, 2503-15.

Loher F. Schmall K. Freytag P. Landauer N. Hallwachs R. Bauer C. Siegmund B. Rieder F. Lehr HA. Dauer M. Kapp JF. Endres S. Eigler A. The specific type-4 phosphodiesterase inhibitor mesopram alleviates experimental colitis in mice. J Pharmacol. Exp. Therap. 305(2):549-56, 2003

Loske, C., Neumann, A., Cunningham, A.M., Nichol, K., Schinzel, R., Riederer, P., and Munch, G., 1998. Cytotoxicity of advanced glycation end products is mediated by oxidative stress. J. Neural Transm. (Budapest) 105, 1005-1015.

Matsumori A. Nunokawa Y. Sasayama S. Pimobendan inhibits the activation of transcription factor NF-kappaB: a mechanism which explains its inhibition of cytokine production and inducible nitric oxide synthase. Life Sci. 67(20):2513-9, 2000

Malinin, N.L., Boldin, M.P., Kovalenko, A.V., and Wallach, D., 1997. MAPK-related kinase involed in NF-늽B induction by TNF, CD95 and IL-1. Nature 385, 540-544.

Mohamed, A.K., Bierhaus, A., Schiekofer, S., Tritschler, H., Ziegler, R., Nawroth, P.P., 1999. The role of oxidative stress and NF-kappa B activation in late diabetic complications. Biofactor 10, 157-167.

Simm, A., Munch, G., Seif, F., Schenk, O., Heidland, A., Richter, H., Vamvakas, S., and Schinzel, R., 1997. Advanced glycation end products stimulate the MAP-kinase pathway in tubulus cell line LLC-PK1. FEBS Lett. 410, 481-484.

Stawowy P. Goetze S. Margeta C. Fleck E. Graf K. LPS regulate ERK1/2-dependent signaling in cardiac fibroblasts via PKC-mediated MKP-1 induction. Biochem Biophys Res Commun 303(1):74-80, 2003

Yoshizaki T., Maegawa H., Egawa K., Ugi S., Nihio Y., et al (2004). Proteinphosphatase 2C as a positive regulator of insulin sensitivity through direct activation of phosphatidylinositol 3-kinase in 3T3-L1 adipocytes. J. Bio. Chem. 279:22715-22726.

Teixeira MM. Gristwood RW. Cooper N. Hellewell PG.Phosphodiesterase (PDE)4 inhibitors: anti-inflammatory drugs of the future?. Trends in Pharmacol Sc. 18(5):164-71, 1997

Yin, M.J., 1998. HTLV-1 Tax protein binds to MEKK1 to stimulate I늽B kinase activity and NF-늽B activation. Cell 93, 875-884.

Yoshikawa M. Suzumura A. Tamaru T. Takayanagi T. Sawada M.Effects of phosphodiesterase inhibitors on cytokine production by microglia.Multiple Sclerosis. 5(2):126-33, 1999