Biology of Adult Human Microglia Studied *in vitro* and *in situ*: Immune Accessory and Effector Functions

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December, 1993

A thesis submitted to the
Faculty of Graduate Studies and Research
in partial fulfilment of the requirements for the degree of:

Doctor of Philosophy

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Abstract

This thesis studying the biology of adult human derived microglia in vitro and in situ, assessed putative immune accessory/effector functions of microglia and correlated in vitro findings with microglia in situ in normal and multiple sclerosis brain specimens. Initial studies describe an isolation protocol for deriving enriched microglial cultures and demonstrate using phenotypic markers of cells of the myeloid lineage, that microglia can be distinguished from peripheral blood monocytes in vitro. Microglia but not astrocytes could function as an antigen presenting cell (APC) for primary and secondary T cell responses. Investigating the basis for the difference in antigen presentation capacity between microglia and astrocytes, it was found that microglia expressed mRNA and protein for IL-1α, IL-6, and TNFα, while astrocytes only had transcript and protein for IL-6. Human recombinant IL-1α, or microglia at non-T cell stimulatory concentrations added to astrocyte T cell co-cultures resulted in the reversal of the inability of astrocytes to present antigen in the MLR. In terms of immune accessory/adhesion molecules, both microglia and astrocytes expressed LFA-1 and ICAM-1 but microglia alone expressed B7/BB-1. Antibody against B7/BB-1 and a CTLA-4 Ig fusion protein that binds to B7/BB-1 partially inhibited the ability of microglia to present antigen. B7/BB-1 expression was also demonstrated on microglia in active MS lesions. In addition to immune accessory/adhesion molecules, microglia expressed FcR I-III in vitro and in situ, and the level of FcR expression on microglia was upregulated in MS brain. Functionally microglia could bind, phagocytose, and lyse antibody coated target cells via FcR dependent mechanisms. Ligation of FcR on microglia resulted in activation of microglia as seen by increased oxidative burst activity and cytokine production. The phagocytosis of myelin by microglia resulted in a similar oxidative burst and cytokine production response. These data indicate that microglia can function in vitro and in situ as immune accessory/effector cells linking the cellular and humoral arms of the immune system, possibly contributing to the pathology seen in multiple sclerosis.

Résumé

Cette thèse évalue les fonctions immunitaires accessoires et effectrices des cellules microgliales d'origine humaine et établit la corrélation entre les données obtenues in vitro et les études in situ sur la microglie de cervaux humains normaux et de cervaux de patients atteints de sclérose en plaques. Les premières études décrivent un protocole d'isolement de cultures enrichies de microglie, et démontrent qu'avec l'utilisation de marqueurs phénotypiques pour les cellules myéloïdes, la microglie peut être différenciée des monocytes in vitro. Les cellules microgliales ont un niveau d'expression du CMH de classe II plus élevé que les astrocytes autologues, au niveau basal et au niveau atteint après induction par l'interféron γ. De plus, les cellules microgliales, et non les astrocytes, peuvent fonctionner en tant que cellules présentatrices d'antigènes pour l'induction de réponses primaires et secondaires de cellules T. En poursuivant l'étude de la capacité à présenter l'antigène de la microglie et des astrocytes, nous avons trouvé que les deux types cellulaires expriment les molécules d'adhésion accessoires LFA-1 et ICAM-1, mais seule la microglie exprime l'antigène B7/BB1. La capacité des cellules microgliales à présenter les antigènes est partiellement inhibée après addition d'anticorps dirigés contre l'antigène B7/BB1. Les cellules microgliales expriment l'ARNm et les protéines pour les cytokines IL-1α, IL-6, et TNFα, tandis que les astrocytes n'expriment que l'ARNm et la protéine IL-6. La capacité des astrocytes à présenter les antigènes dans une réaction lymphocytaire mixte est inversée par l'addition d'IL-1α recombinante humaine ou de microglies à une concentration non-stimulante pour les cellules T. En plus de l'expression du CMH de classe II, la microglie exprime les récepteurs Fc I à III, in vitro et in situ, et les niveaux d'expression les plus élevés sont observés dans les lésions actives de sclérose en plaques. Les cellules microgliales peuvent fixer, phagocyter, et lyser des cellules de cibles opsonisées. La génération d'une poussée oxidative et de cytokines est observée après liaison des cellules cibles opsonisées. La phagocytose de la myéline par la microglie produit une réaction semblable. L'ensemble de ces données indique que les cellules microgliales peuvent fonctionner in vitro et in situ en tant que cellules immunitaires effectrices et accessoires et peuvent, de ce fait, contribuer à la pathologie observée dans la sclérose en plaques.

Acknowledgments

The author is indebted to the countless hours spent in discussion and in the laboratory with Jack P Antel M.D., and Voon Wee Yong, Ph.D., who in addition to providing valued support and friendship also served as superb academic role models.

The author is also indebted to Elling Ulvestad, a collaborator and friend with whom I have spent several hours discussing the biology of microglia.

The author would also like to acknowledge the patience and understanding of his family during his continued absence from home.

Specific sections of this thesis represent collaborative efforts involving individuals both in the neuroimmunology laboratory and elsewhere. The following acknowledgement pertains to work performed by other individuals that is presented in this thesis.

- Amit Bar-Or for performing initial studies of the rate of survival of microglia versus peripheral blood monocytes in Chapter 1 and Andre Olivier, a Neurosurgeon, for providing neurosurgical tissue specimens used in Chapter 1.
- Elling Ulvestad a co-author on Chapters 2-9 who performed in situ staining of pathology materials and prepared immune complexes and opsonized target cells, data presented in Chapter 7. Elling Ulvestad and I were equal contributors to the data and writing of Chapter 7 and therefore share authorship as is indicated in the manuscript.
- Manon Blain, a technician in the laboratory of Dr Jack Antel, for isolation and purification of $CD4^+$ T cells used in Chapter 4 and 5.
- Nora Dooley, a graduate student in the laboratory of Dr Voon Wee Yong, for initial instruction on RNA extraction and RT-PCR analysis of cytokine gene expression. RT-PCR data presented in Chapter 5. RT-PCR studies reported in the thesis were performed by the author.
- Anders Waage, from Microbiology and Immunology, University of Bergen, whose laboratory performed cytokine bioassays in Chapters 5 and 9.
- JoAnne McLaurin, a post-doctoral fellow in the laboratory of Dr. Jack Antel, for isolation and purification of human myelin and preparation of myelin vesicles in Chapter 9.

Table of Contents

Part II: Immune accessory functions

Chapter 4

Chapter 5

Chapter 6

Part III: Immune effector functions

Chapter 7

Chapter 8

Chapter 9

Page

Part IV: Conclusion

Chapter 10

List of figures

List of Tables

Thesis Overview

The following thesis is divided into 4 parts; the first 3 sections constitutes original work performed describing the biology of adult human derived microglia in vitro, assessing putative immune accessory and effector functions of microglia in vitro, and correlating in vitro, data with MS pathology material.

Part I describes the isolation of microglia from adult human brain, defining the biology of these cells compared to peripheral blood monocytes and autologously derived astrocytes, and the ability of microglia to function as antigen presenting cells. In vitro data on microglia phenotype were confirmed in situ, in MS brain sections.

Part II and III define the ability of microglia to function as immune accessory cells to stimulate unprimed and previously activated T cells, and the ability of microglia to function as an effector cell in immune mediated antibody dependent mechanisms. When possible, functional comparisons were made between microglia and either monocytes or astrocytes.

Part IV provides a brief summary of the data presented in the thesis as it might be related to MS, and concludes with a summary of the original work contained herein.

This thesis, in accordance with the "Guidelines concerning thesis preparation " of the Faculty of Graduate Studies and Research, consists of papers that have already been published or which have been submitted for publication. Each chapter in the thesis excluding Chapter 10, was written as manuscript for publication and is either published, in press, or submitted (outlined in Chapter 10). I am the first author of Chapters 2, 4, 5, 6, and 9, and the second author of Chapters 3, 7, and 8. Elling Ulvestad and myself contributed equally to the data and writing of the manuscript resulting in Chapter 7. Each chapter has both an introduction and discussion of the work as it relates to current and past literature. The following introductory chapter is included not as a review of the literature, but as an introduction to the assumptions made in the body of the thesis and as a basis of the questions asked and work performed.

Chapter 1

Introduction

Multiple sclerosis

The major issues pursued in this thesis relate to the postulated role of microglia in contributing to the development of autoimmune disease of the adult human central nervous system. This role is postulated to reflect the interaction between microglia and both the cellular and humoral arms of the immune system. This thesis investigates the contribution of microglia as regulators and effector cells of immune responses within the CNS. Multiple sclerosis (MS), characterized clinically by an apparent spontaneous onset and a relapsing/progressive course, is postulated to be an autoimmune disease (1,2). Pathologically, MS is characterized by multifocal lesions within the CNS with perivascular inflammatory infiltrates, astrogliosis, demyelination, numerous macrophages containing myelin debris, and reactive microglia. Lesions in MS are of different ages and activity correlating with the observed chronic and relapsing clinical course of the disease. The putative target of the inflammatory response in MS is against the oligodendrocyte and/or its myelin membrane. Experimental allergic encephalomyelitis (EAE), a T cell mediated animal disease induced by active immunization of the host animal with antigen in adjuvant emulsion (3) or by the passive transfer of myelin sensitized $CD4^+$ T cells to nonimmune recipients (4,5), provides a model of immune mediated CNS disease from which parallels to MS have been drawn.

Both T and B lymphocytes are implicated as cells contributing to the development of MS and EAE central nervous system (CNS) lesions (6). With the development of monoclonal antibodies against T cell subsets, it has been demonstrated that many of the lymphocytes in early MS lesions are MHC class II restricted, $CD4^+$ T cells (7). Based on these early studies and on clinical histopathological studies in EAE it is believed that the presence of infiltrating $CD4^+$ T cells in the CNS of MS patients correlates with acute and active disease (8,9). Subsequent studies of T cell phenotype in MS and EAE lesions

demonstrated an accumulation of T cells with increased Ta_1 and IL-2 receptor expression, and CD45 low or CD45RO phenotype, suggesting that the T cells in the CNS have undergone differentiation or are activated (10-13). These data suggest that T cells in MS and EAE are actively engaged in an immune effector function. While the antigen(s) to which the immune response is directed are known in the EAE model, putative antigen(s) in MS are less well defined, though myelin components are implicated. Myelin basic protein reactive T cells have been identified in the blood of normal individuals (14) and elevated frequencies have been reported in the blood and cerebral spinal fluid (CSF) of MS patients (15). Additionally, CNS and CSF derived T cells exhibit a limited heterogeneity in the rearrangement of their T cell receptors (TCR) (16-18) possibly the result of enrichment of T cells of similar specificities. Whether T cell sensitization to myelin antigens in MS occurs in the periphery or in the CNS remains speculative. The conditions under which T cells gaining access to the CNS are subsequently stimulated to proliferate or become anergic or apoptotic remains to be defined. Resident CNS antigen presenting cells may be important determinants of these outcomes.

In the normal CNS T lymphocytes are rarely found (19-21). A critical factor for T cells to gain access to the CNS is their level of activation. Activated T cells, irrespective of MHC class II restriction and antigen specificity, gain entry into the CNS (22-24). Whether T cells entering the CNS remain, is dependent on the ability of the T cell to see its antigen. $CD4^+$ T cells cannot respond to antigens in the soluble from. The antigens must be processed and presented to the lymphocyte in the context of the correct self MHC molecules (25,26). Therefore, a requirement for antigen presentation in the CNS by resident presenting cells would be the expression of MHC class II molecules and additional immune accessory/adhesion molecules. The CNS of healthy animals has been repeatedly demonstrated to lack of MHC class II expression (27,28). In MS, expression of MHC class II molecules has been reported on brain capillaries (29), and on astrocytes (30) and microglia (31). Early studies demonstrating MHC class II expression in the CNS were marred by the limitations of identifying cell types morphologically and by the inability to perform double labelling experiments. In the rodent CNS, the levels of MHC class II expression have been demonstrated to be upregulated or induced following IFN gamma injection (32) or in

response to viral infection (33) Expression of MHC class II antigens on microglia in histologically normal human brain has been demonstrated (34,35).

In addition to the involvement of T cells in MS lesions, B cells have also been implicated to play a role. Indeed, increased intrathecal immunoglobulin (IgG) synthesis is a hallmark of MS (36,37) and is also seen in EAE (38,39). In addition to increased intrathecal oligoclonal IgG synthesis in MS, there is evidence of blood brain barrier (BBB) damage with resultant deposition of IgG, immune complexes, and complement (40,41) all implicated to play a role in the CNS immune mediated pathology. The combined effects of T and B cells contributing to the pathology seen in MS and EAE have been demonstrated in studies where a combination of anti MOG antibody directed against surface molecules on oligodendrocytes, and passively transferred T cells, result in a significant increase in disease activity over that induced by T cells or antibody alone (42,43).

The production of cytokines in MS and EAE have been studied both to ascertain the level of T cell activation in the parenchyma and to delineate the possibility of injury or activation of individual CNS glial cells as a result of increased cytokine levels in the CNS. These studies have demonstrated elevated IL-2 (44,45) IL-1, TNFα, and lymphotoxin in MS tissues (46,47) Neutralizing antibodies against TNFα and IL-1α have been demonstrated to abrogate disease symptoms in EAE (48,49). The glial cells synthesizing and secreting cytokines in MS and EAE have not been definitively demonstrated.

The above immune-pathologic observations in MS and EAE suggest a state of activation of inflammatory cells and glial cells corresponding to phases of active lesion formation. The contribution of microglial cells in the regulation of immune reactivity and effecting tissue directed responses form the basis of the work presented in this thesis.

Microglia

The central nervous system (CNS) glial cells are composed of macroglia; oligodendrocytes and astrocytes that are derived from neuroectodermal tissues, and microglia. The origin and indeed the very existence of microglia as a unique cell type in the CNS, has been the subject of considerable debate (for review see 50-52). Microglia, until just recently, possibly the result of not having a cell specific marker, have received little investigative

attention as compared to astrocytes and oligodendrocytes. The development of tissue culture protocols for the isolation of fetal rodent (53-55) and fetal human microglia (56-58), in parallel with increased antibody and lectin staining technology (59-63), has resulted in increased information concerning microglia lineage and putative microglial functions. Despite these advances questions concerning microglial lineage and function remain (for review see (64). Contributing to the confusion concerning the role of microglia in human CNS disease, is the apparent species differences in function and phenotype between rodent and human microglia. Thus rodent microglia have detectable levels of CD4 expression (65) while human microglial cells do not (66). Rodent microglia are non specific-esterase (NSE) positive cells (67) while adult human microglia are NSE negative (68). Additionally, a number of monoclonal antibodies recognizing rodent microglia including ED2 (60), and F4/80 (62) do not cross species to human. Human microglial cells express many antigens also demonstrated on myeloid cells (69,70) making it difficult to distinguish between infiltrating monocytes and macrophages and resident parenchymal microglia in inflammatory CNS diseases.

Microglia subtypes and origin

The microglia population within the CNS has been classified into two major morphologic subtypes consisting of ameboid (including uni-polar and bi-polar cells) and ramified cells. Microglia juxtaposed with the CNS vasculature are termed perivascular microglia (71) and are ameboid in shape. Parenchymal microglial cells in the developing CNS are ameboid and in the adult CNS ramified microglia appear uniformly throughout the neuropil, forming an extensive reticular array. Ameboid microglia are found in the adult CNS in response to injury and inflammation. In tissue culture studies, the majority of cells obtained are ameboid (53-55).

While there exist controversy concerning the embryonic origin of microglia (72-75) developmental studies using immunocytochemical and enzymatic characterization have demonstrated that microglia are most likely haematogenous cells of monocytic origin (74,76,77). Other theories of microglial origination include the belief that microglia are derived from neuroectodermal matrix cells together with macroglia (78) and that microglia

are derived from pericytes (79). Contributing to the difficulty in assessing the origination of CNS microglia might be differences in experimental methodologies, and more importantly defining the cell population in the CNS that are considered to be brain macrophages/microglia.

Ameboid microglia

Rio-Hortega (1932) (80) thought that ameboid microglia were formed from embryonic corpuscles from the pia that migrate into the CNS. He also noted that microglia might be derived from the blood mononuclear cells. Immamoto and Leblond (1978) (81) first described that ameboid microglia cells in postnatal corpus callosum ultrastructurally resembled monocytes. Following the injection of carbon into the peripheral circulation of 3-5 day old rats, Ling (1979) (82) unequivocally demonstrated that carbon-labelled ameboid microglia were representative of monocytes from the blood that had ingested the carbon in the vasculature and had then infiltrated the post-natal brain to establish residence in the corpus callosum. Immunohistochemical methods have added further evidence of the monocytic origin of ameboid microglia. These studies include data using anti-granulomonocytic serum (83), using antibodies against the Fc portion of immunoglobulin and complement type three receptors (74), and the demonstration of leukocyte common antigen and major histocompatibility complex antigens on microglia (84).

Ramified microglia

Hortega further postulated that ramified microglia represent a transition from the ameboid microglia . Hortega (1932) (80) postulated that cells with argentophilic cytoplasm first appeared in the pia and then further penetrated the CNS changing from a globose to an ameboid form followed by the formation of branched processes and the transition to a ramified cell. That ramified microglial cells are derived from mesodermal origin is widely accepted though a contrary view that these cells are derived from neuroectodermal cells of the subependyma subjacent to the lateral ventricles is also held (85,86)

Rodent ameboid microglia express several monocyte associated markers that are apparently down regulated on ramified microglia. This observation has lead Perry and Gordon (74) to consider the ramified rodent microglial cells to be "switched off

macrophages". Though developmental studies describe the transformation of ameboid microglia to bi-polar and then ramified cells in situ the possibility has been raised that ramified microglial cells can migrate directly from the peripheral blood (87). Dunning and Furth (88) studying cultured cells in vitro documented that cultured blood derived monocytes can transform into cells identical to ramified microglia.

Hickey and Kimura (89) using bone marrow chimeric animals were able to demonstrate donor-specific antigens in the host brain associated with both the majority of perivascular cells but labelling a small percentage of parenchymal microglia. More recent evidence using the same chimeric model suggests that the perivascular cells have a constant rate of turnover and replacement by blood derived cells while the parenchymal ramified microglia have a low rate of turnover.

Microglial functions

The majority of early studies related to the microglial response in disease or injury in situ, focused on morphologic changes as an indicator of activation. More recent studies using immunocytochemical methods analyzing the expression of receptors for the constant region of immune globulin (FcR), complement type 3 receptors, and MHC class II antigens, describe microglia activation in a variety of human diseases including Alzheimer's disease (90) Parkinson's disease (91) and in MS (92).

More recently, microglia have been implicated as central contributors involved in CNS immune surveillance and activation (93,94), in the mediation of human immunodeficiency virus (HIV) CNS pathology (95) and activation and pathology in brain injury models (96,97).

This thesis sought to develop our knowledge concerning the possible role of microglia contributing to CNS immune reactivity. Specifically, this thesis sought to define the ability of microglia to function as an immune accessory cell contributing to T cell activation; and the ability of microglia to function as an immune effector cell through the interaction of FcRs on microglia. Comparisons of microglia phenotype and function have been made with peripheral blood mononuclear cells and astrocytes. When possible, in vitro findings have been confirmed in situ using CNS tissue from non-inflammatory cases and from MS brain.

References

1. Waksman BH and WE Reynolds. 1984. Minireview: Multiple sclerosis as a disease of immune regulation. Proc Soc Exp Bri Medicine. 175:282.

2. McFarlin DE and H McFarland. 1982. Multiple sclerosis. N Eng J Med. 307:1188.

3. Paterson PY. 1981. Current prospectives in neuroimmunological disease: multiple sclerosis and experimental allergic encephalomyelitis. Clin Immunol Rev. 14:581.

4. Patterson PT. 1960. Transfer of allergic encephalomyelitis in rats by lymph node cells. J Exp Med. 111:119.

5. Ben-Nun A, H Wekerle, and IR Cohen. 1981. The rapid isolation of clonable antigen specific T lymphocyte lines capable of mediating EAE. Eur J Immunol. 11:195.

6. Traugott U, Shevach E, Chiba J, Stone JH, and CS Raine. 1981. Autoimmune encephalomyelitis: simultaneous identification of T and B cells in the target organ. Science. 79:173.36.

7. Booss J, Esiri M, Tourtellote WW, and DY Mason. 1983. Immunohistochemical analysis of T lymphocyte subsets in the central nervous system in chronic progressive multiple sclerosis. J Neurol Sci. 62:219.

8. Brown A, McFarlin DE, and CS Raine. 1982. The chronological neuropathology of relapsing experimental allergic encephalomyelitis in the mouse. Lab Invest. 46:171.

9. Raine CS. 1990. Demyelinating diseases. In: Textbook of neuropathology. Ed. RL Davies and DM Robertson (eds). Williams and Wilkins:Baltimore. 468.

10. Hafler DA, Buchsbaum M, Johnson D, and H Weiner. 1985. Phenotypic and functional analysis of T cells cloned directly from the blood and cerebrospinal fluid of patients with multiple sclerosis. Ann Neurol. 18:451.

11. Sobel RA, Hafler DA, Castor EM, Morimoto C, and HL Weiner. 1988. The 2H4 (CD45R) antigen is selectively decrease in multiple sclerosis lesion. J Immunol. 140:2210.

12. Bellamy AS, Calder VL, Freedman M, and A Davison. The distribution of interleukin-2 receptor bearing lymphocytes in multiple sclerosis: evidence for a key role of activated lymphocytes. Clin Exp Immunol. 61:248.

13. Hoffman FM, Von Hanwehr RI, Dinarello CA, Mizell SB, Hinton D, and JE

Merril.1986. Immunoregulatory molecules and IL-2 receptors identified in multiple sclerosis brain. J Immunol. 136:3239.

14. Burns J., A Rosenzweig, B Zweiman, and RP Lisak. 1983. Isolation of myelin basic protein reactive T cell lines from normal blood. Cell Immunol. 81:438.

15. Olsson T, WW Zhi, B Hojeberg, V Kostulas, J Yu-Ping, G Anderson, HP Ekre, and H. Link. 1990. Autoreactive T lymphocytes in multiple sclerosis determined by antigen induced secretion of interferon gamma. J Clin Invest. 86:901.

16. Wucherpfennig KW, J Newcombe, H Li, C Keddy, ML Cuzner, and DA Hafler. 1992. T cell receptor V alpha-V beta receptor and cytokine gene expression in active multiple sclerosis lesions. J Exp Med. 175:993.

17. Oksenberg JR, S Stuart, AB Begovich, RB Bell, HA Erlich, L Steinmann, and CCA Bernard. 1990. Limited heterogeneity of rearranged T cell receptor V alpha transcripts in brains of multiple sclerosis patients. Nature 345:344.

18. Ben-Nun A, RS Liblau, L Cohen, D Lehmann, E Tournier-Lasserve, A Rosezweig, Z Jinguv, J Rass, and MA Bach. 1991. Restricted T cell receptor V beta gene usage by myelin basic protein specific T cell clones in multiple sclerosis: predominant genes vary in individuals. Proc Natl Acad Sci (USA). 88:2466.

19. Hauser SL., AK Bhan, G Giles, CJ Hoban, EL Reinherz, and HL Weiner. 1983. Immunohistochemical staining of human brain with monoclonal antibodies that identify lymphocytes, moncytes and Ia antigen. J Neuroimmunol. 5:197.

20. Hickey WF, JA Cohen, JB Burns. 1987. A quantitative immunohistochemical comparison of actively versus adoptively induced experimental allergic encephalomyelitis in the lewis rat. Cell Immunol. 109.

21. Matsumoto Y, M Fojiwara. 1988. Adoptively transferred EAE in chimeric rats: identification of transferred cells in the lesions of the central nervous system. J of Neuroimmunol. 65:23.

22. Hickey WF, BL Hsu, and H Kimura. 1989. T cell entry into the rat central nervous system. FASEB 3:A482.

23 Meyerman R, T Korr, and H Werkerle. 1986. Specific target retrieval by encephalitogenic T cell lines. Clin Neuropath. 5:101.

24. Wekerle H, D Sun, RL Oropiza-Wekerle, and R Meyerman. 1987. Immune reactivity in the nervous system: modulation of T lymphocyte activation by glial cells. J Exp Bio. 132:43.

25. Allen PM. 1982. Antigen processing at the molecular level. Immunol Tod. 8:270.

26. Unanue ER, PM Allen. 1987. The basis for immunoregulatory role of macrophages and other accessory cells. Science. 236:551.

27. Sobel RA, BW Blanchette, AK Bhan, and RB Colvin. 1984. The immunopathology of experimental allergic encephalomyelitis. II. Endothelial cell Ia increases prior to inflammatory cellular infiltrates. J Immunol. 134:233.

28. Vass K, H Lassmann, HM Wekerle, HM Wisiniewski. 1986. The distribution of Ia antigens in the lesions of acute EAE. Acta Neuropath. 70:149.

29. Traugott U, and CS Raine. 1985. Multiple sclerosis. Evidence for antigen presentation in situ by endothelial cells and astrocytes. J Neurol Sci 69:365.

30. Traugott U, Scheinberg LC, and CS Raine. 1985. On the presence of Ia-positive endothelial cells and astrocytes in multiple sclerosis lesion and its relevance to antigen presentation. J Neuroimmunol. 8:1.

31 Boyle EA and PL McGeer. 1990. Cellular immune response in multiple sclerosis plaques. Am J Pathol. 137:575.

32. Wong GMW, Bartlett PF, Clark-Lewis I, Battye F, and JN Schrader. 1984. Inducible expression of H-2 and Ia antigens on brain cells. Nature. 310:688.

33. Massa PT, and V ter Meulen. 1987. Analysis of Ia induction on lewis rat astrocytes in vitro by virus particles and bacterial adjuvants. J Neuroimmunol. 13:239.

34. Lampson LA, and WF Hickey 1986. Monoclonal antibody analysis of MHC expression in human brain biopsies: tissue ranging from histologically normal to that showing different levels of glial tumor involvement. J Immunol. 136:4054.

35. Graeber MB, Streit WJ, Buringer T, Sparks DC, and GW Kreutzberg. 1992. Ultrastructural location of major histocompatibility complex class II positive perivascular cells in histologically normal human brain. J Neuropath Exp Neurol. 51:303.

36. Tourtollotte WW, and MJ Walsh. 1984. Cerebrospinal fluid profile in multiple sclerosis. In: CM Posner, DW Paty, L Scheinberg, W McDonald, and GC Ebers (EDS) The diagnosis

of multiple sclerosis. Thieme-Stratton, New York, 165.

37. Xiao BG, Linnington C, Link H. 1991. Antibodies to myelin-oligodendrocyte glycoprotein in cerebrospinal fluid from patients with multiple sclerosis and controls. J Neuroimmunol. 31:91.

38. Whitacre CC, DH Mattson, PY Paterson, RP Roos, DJ Paterson, and BGW Arnason. 1981. Cerebrospinal fluid and serum oligoclonal IgG bands in rabbits with experimental allergic encephalomyelitis. Neurochem Res. 6:87.

39. Whitacre CC, DH Mattson, ED Day, DJ Patterson, RP Roos, and BGW Arnason. 1982. Oligoclonal IgG in rabbits with experimental allergic encephalomyelitis: Non- reactivity of the bands with sensitizing neural antigens. Neurochem Res. 7:1209.

40. Gay B, and M Esiri. 1977. Immunoglobulin containing cells in multiple sclerosis lesions. Lancet. 2:478.

41. Gay D, and M Esiri. 1991. Blood-brain barrier damage in acute multiple sclerosis plaques. Brain. 114:557.

42. Linington C, M Bradl, H Lassmann, C Brunner, K Vass. 1988. Augmentation of demyelination in rat acute allergic encephalomyelitis by circulating mouse monoclonal antibodies directed against a myelin/oligodendrocyte glycoprotein. Am J Pathol. 130:443.

43. Linington C, H Lassmann. 1987. Antibody response in clinical relapsing experimental allergic encephalomyelitis: correlation of serum demyelinating activity with antibody titre to myelin/oligodendrocyte glycoprotein (MOG). J Neuroimmunol. 17:61.

44. Selmaj K, Plater-Zyberk C, Rockett KA, Maini RN, Alam R, Perkin GD, and FC Rose. 1986. Multiple sclerosis: increased expression of IL-2 receptors on lymphocytes. Neurology. 36:1392.

45. Trotter JL, Collins KG, and RC van der Vew. 1991. Serum cytokine levels in chronic progressive multiple sclerosis: interleukin-2 levels parallel tumor necrosis factorα levels. J Neuroimmunol. 33:29.

46. Hauser SL, Doolittle TH, Lincoln R, Brown RH, and CA Dinarello. 1990. Cytokine accumulation in CSF of multiple sclerosis patients: frequent detection of interleukin-1 and tumor necrosis factor but not interleukin-6. Neurology 40:1735.

47. Selmaj K, Raine CS, Canella B, and CF Brosnan. Identification of lymphotoxin and

tumor necrosis factor in multiple sclerosis. J Clin Invest. 87:949.

48. Ruddle NH, Bergman CM, McGrath KM, Lingenheld EG, Grunnet ML, Padula SJ, and 2RB Clark. 1990. An antibody against lymphotoxin and tumor necrosis factor prevents transfer of experimental allergic encephalomyelitis.

49. Jacobs CA, PE Baker, ER Roux, KS Picha, B Toivola, S Wagh and MK Kennedy. 1991. Experimental autoimmune encephalomyelitis exacerbated by IL-1α and suppressed by soluble IL-1 receptor. J Immunol. 146:2983.

50. Jordan FL, WE Thomas. 1988. Brain macrophages: question of origin and interrelationships. Brain Res Rev. 131:165.

51. Ling EA. 1989. The origin and nature of microglia: In Advances in cellular neurobiology. Vol 2. S Federoff (ed), Hertz L. New York: Academic press.

52. Perry VH, S Gordon. 1988. Macrophages and microglia in the nervous system. Trend Neurosci. 11:273.

53. Frei K, Siepl C, Groscurth, S Bodmer, and A Fontana. 1988. Immunobiology of microglial cells. In: Annals of the new york academy of sciences . 540:218.-

54. Gebicke-Haerter PJ, J Bauer, A Schobert, H Northuff. 1989. Lipopolysaccharide-free conditions in primary astrocyte cultures allow growth and isolation of microglial cells. J Neurosci. 9:183.

55. Giulian D, and TJ Baker. 1986. Characterization of ameboid microglia isolated from developing mammalian brain. J Neurosci. 6:2163.

56. Peudenier S, Hery C, Montagnier L, and M Tardieu. 1991. Human microglial cells: characterization in cerebral tissue and primary culture, and study of their susceptibility to HIV-infection. Annal Neurol. 29:152.

57. Sebire G, D Emilie, C Wallon, C Hery, O Deverge, JF Delfraisy, P Galland and M Tardieu. 1993. in vitro production of IL-6, IL-1ß, and tumor necrosis factorα by human embryonic microglia and neural cells. J Immunol 150:1517.

58. Lee SC, W Liu, CF Dickson, CF Brosnan, and JW Berman. 1993. Cytokine production by human fetal microglia and astrocytes. Differential induction by lipopolysaccharide and IL-1 beta. J Immunol. 150:2659.

59. Boya J, Calvo JL, Barbonell AL, and A Borregona. 1991. A lectin histochemical study

on the origin of rat microglial cells. Act Anat. 130:329.

60. Graeber MB, WJ Streit, and GW Kreutzberg. 1989. Identity of ED-2 positive perivascular cells in rat brain. J Neurosci Res. 22:103.

61. Mannoji H, H Yeger, LE Becker. 1986. A specific marker (lectin ricinus communis agglutinin I) for normal human microglia and application to routine histopathology. Acta Neuropathol. 71:341.

62. Hume DA, VH Perry, and S Gordon. 1984. The mononuclear phagocyte system of the mouse defined by immunohistochemical localization of antigen F4/80. Macrophages associated with epithelia. Anat Rec . 210:503.

63. Perry VH, DA Hume, S Gordon. 1987. Modulation of CD4 antigen on macrophages and microglia in rat brain. J Exp Med. 166:1138.

64. Theele DP and WJ Streit. 1993. A chronical of microglial ontogeny. Glia 7:5.

65. Crocker PR, Jeffried WA, Clark SJ, Chung LP, and S Gordon. Species heterogeneity of macrophage expression of the CD4 antigen. J Exp Med 166:613.

66. Watkins BA, Dorn HH, Kelly WB, Armstrong RG, Putts BJ, Micjaels F, Kufa CV, and M Dubois-Dalq. 1990. Specific tropism of HIV for microglial cells in primary brain cultures. Science 249:549.

67. Ling EA. Influence of cortisone on ameboid microglia and microglial cells in the corpus callosum in post natal rats. J Anat. 134:705.

68. Hayes GM, Woodroofe MN, and ML Cuzner. 1988. Characterization of microglia isolated from adult human and rat brain. J Neuroimmunol 19:178.

69. Esiri MM, Izzi MS, and MC Reading. Macrophages, microglial cells, and HLA-DR antigens in fetal and infant brain.

70. Gould SJ and S Howard. An immunohistochemical study of macrophages in the HIV fetal brain. Neuropath and Applied Neurobiology. 17:383.69. Cammermeyer J. 1970. The life history of the microglial cells. J Anat. 175:229.

71. Hickey WF. and H Kimura. 1988. Perivascular microglial cells of the CNS are bone marrow derived and present antigen in vivo. Science 239:291.

72. Cammermeyer J. 1970. The life history of the microglial cells: A light microscopic study. Neurosci Res. 3:43.

73. Oehmichen M, Wietholter H, Greales MF. 1979. Immunological analysis of human microglia, lack of monocytes and lymphoid differentiation antigens. J Neuropath Exp Neurol. 38:99.

74. Perry VH and S Gordon. 1988. Macrophages and microglia in the nervous system. Trend Neurosci. 11:273.

75. Schleper RL, and EK Adrian. 1986. Monocytes become macrophages, they do not become microglia: a light and electron microscopic autoradiographic study suing ^{125}-Iododeoxyuridine. J Neuropath Exp Neuro. 45:1.

76. Boya J, J Calvo, AL Carbonell, E Garcia-Maurino. 1986. Nature of macrophages in rat brain: a histochemical study. Acta Anat. 127:142.

77. Boya J, AL Carbonell, J Calvo, A Borregon. 1987. Ultrastructural study on the origin of rat microglial cells. Acta Anat 130:329.

78. Fujita S, and T Kitamura. 1976. Origin of brain macrophages and the nature of the microglia. In: Progress in neuropathology. Zimmerman HM (ed). Vol III. Grume and Stratton:New York, 1.

79 Mori S, and CP Leblond. 1969. Identification of microglia in light and electron microscopy. J Comp Neurol. 135:57.

80. Rio-Hortega. 1932. Microglia. In: cytology and cellular pathology of the nervous system. VOl 2. W Penfield (ed). Paul Hoeber, New York, 481.

81. Imamoto I, and CP Leblond. 1978. Radio autographic investigation of glial genesis in the corpus callosum of young rats. II. origin of microglial cells. J Comp Neurology.

82. Ling EA. 1979. Transformation of monocytes into ameboid microglia and into microglia in the corpus callosum of postnatal rats as shown by labelling monocytes by carbon particles. J Anat. 128:847.

83. Miyake T, Tsuchihashi Y, Kitamum T, and S Fujita. 1984. Immunohistochemical studies of blood monocytes infiltrating into neonatal rat brain and developing mouse brain. Neurosci 15:313.

84. Ling EA, Kaur C, and WC Wong. 1991. Expression of major histocompatibility complex and leukocyte common antigens in ameboid microglia in post natal rats. J Anat. 177:117.

85 Vaughn JE. 1969. An electron microscopic analysis of gliogenesis in rat optic nerves. Z Zellforsch Mikrosk Anat. 94:293.

86. Vaughn JE, and A Peters. 1968. A third neuroglia cell type. An electron microscopic study. J Comp Neurol. 133:269.

87. Chugani DC, Kedersh NL, and LH Rome. 1991. Vault immunofluoresence in the brain: new insights concerning the origin of the microglia. J Neurosci. 11:256.

88. Dunning F and J Furth. 1935. Studies in the relation between microglia, histiocytes, and monocytes. Am J Pathol. 11:895.

89. Hickey WF and H Kimura. 1988. Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vitro. Science. 239.

90. Itagaki S, McGeer PL, Akiyama H. 1988. Presence of cytotoxic suppressor and leucocyte common antigen positive cells in Alzheimer disease brain tissue. Neurosci Lett. 91:259.

91. McGeer PL, Itagaki S, and EG McGeer. 1988. Expression of the histocompatibility glycoprotein HLA-DR in Neurologic disease. Acta Neuropath. 76:550.

92. Hayes GM, MN Woodroofe, and ML Cuzner. 1987. Microglia are the major cell type expressing MHC class II in human white matter. J Neurol Sci. 80:25.

93. Streit WJ, Graeber MB, and GW Kreutzberg. 1988. Functional plasticity of microglia: a review. Glia. 1:301.

94. Guilian D, TJ Baker, L Shih, LB Lachamsi. 1986. Interleukin-1 of the central nervous system is produced by ameboid microglia. J Exp Med. 164:594.55.

95. Dickson DW, Mattiace LA, Kure K, Hutchins K, Lyman WD, and CF Brosnan. 1992. Biology of disease: Microglia in human disease with an emphasis on acquired immune deficiency syndrome. Lab Invest 64:135.

96. Giulian D. 1987. Ameboid microglia as effectors of inflammation in the central nervous system. J Neurosci Res. 18:155.

97. Graeber MB, Tetzlaff W, Streit WJ, and Kreutzberg. 1988. Microglia cells but not astrocytes undergo mitosis following rat facial nerve axotomy. Neurosci Lett 85:317.

Part I

Biology of adult human derived microglia in vitro and in situ

Chapter 2

BIOLOGY OF ADULT HUMAN MICROGLIA IN CULTURE:COMPARISONS WITH PERIPHERAL BLOOD MONOCYTES AND ASTROCYTES

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Preface

This chapter describes a protocol for the isolation and enrichment of adult human derived microglia in vitro. To assess the purity of microglial cultures comparisons were made between brain derived microglial cells and peripheral blood monocytes. Characteristics studied included rate of survival in vitro, adherence properties, and the surface and enzymatic phenotype of microglia. Comparisons between autologous microglia and astrocytes were under taken with regard to expression of surface MHC class II molecules. Major points demonstrated in this chapter include:

1. Enriched microglial cultures (>95%) can be established utilizing surgically resected material from adult human brain. Adult human derived microglia, in contrast to fetal rodent and fetal human microglia, can be selectively purified based on their strong adherence to tissue culture plastic.

2. Based upon survival in vitro, adherence properties, and the rate of morphological development, the majority of "microglia" in our culture system are most likely parenchymal derived cells and not monocytes trapped in the vasculature or contaminating the brain specimen.

3. A differentiation pattern was demonstrated that can distinguish between microglia and peripheral blood monocytes in vitro; peripheral blood monocytes were Leu-M3, non-specific esterase, and CD4 positive while microglia were negative for all three markers.

4. Ameboid, bi-polar, and ramified microglia morphologies were demonstrated in vitro, and each cell type expressed MHC class II antigens. The number of microglial cells expressing MHC class II antigens under basal and IFN gamma activation conditions was greater than that of autologous astrocytes.

5. Microglia could function as an antigen presenting cell presenting the recall antigen Candida albicans to freshly derived autologous T cells, resulting in T cell proliferation.

ABSTRACT

We have compared phenotypic and functional properties of surgically-derived human adult microglia to autologous and allogenic peripheral blood-derived monocytes and with astrocytes derived from the same surgical resection. We found that microglia differed from peripheral blood monocytes with respect to adhesion properties and survival rates in vitro. Microglia, similar to resident macrophages in different tissues, expressed many but not all (CD4, Leu-M3, non specific esterase) monocyte/ macrophage associated markers tested, a pattern similar to that of terminally differentiated cells of this lineage. As with other human tissue macrophages, but in contrast to astrocytes, microglia did not undergo DNA synthesis in vitro, assessed using BrdU incorporation. Under basal culture conditions the majority of microglia of all morphologic subtypes (ameboid, bipolar, ramified) expressed MHC class II molecules; by flow cytometric analysis, mean fluorescence intensity of these cells was less than that of blood monocytes (relative to isotype control). In vitro MHC Class II antigen expression on microglia, under basal and interferon gamma (IFN) activating conditions, was greater than astrocytes. Freshly derived T cells cultured with 1-10% autologous microglia plus Candida albicans underwent active proliferation, indicating the functional capacity of the microglia to serve as antigen presenting cells.

INTRODUCTION

Microglial cells were originally defined as a distinct cell type based on their in situ morphology within the central nervous system (CNS). The origin of these cells has most often been ascribed to a bone marrow-derived monocyte/macrophage lineage (1-4), with entry into the CNS during the course of development; others have proposed a non-monocytic origin of these cells (5-6), or ascribed their origin to neuroectoderm (7). Microglia participate in the pathologic reactions of the CNS in response to trauma, inflammation, and neuronal degeneration processes. In such conditions, microglia responses include morphological changes, "proliferation" and expression of major histocompatibility complex (MHC) class II molecules. These responses have been regarded as indicators that the microglial cells are "activated". Among functional properties ascribed to microglial cells are phagocytosis of damaged neural elements (8), antigen presentation to T-lymphocytes which could mediate neuroimmune interaction (3,9), and production of soluble factors (10) which may induce tissue injury (11,12) and gliosis (13).

In vitro tissue culture techniques provide opportunities to assess the properties of microglial cells under "baseline" and "activating" conditions. Given the apparent similarities in both phenotypic and functional properties between microglia and systemic monocytes/macrophages, concern is raised as to whether resident "microglia" or contaminating systemic monocytes/macrophages are being isolated from the CNS in such studies. Suzumura et al (14) referred to the phagocytic cells isolated from newborn mice as macrophage/microglia; this population was positive for non specific esterase (NSE) but peroxidase negative, expressed MHC class I and II molecules only after exposure to interferon gamma (IFN), and did not actively proliferate. These cells could be maintained in culture for at least eight weeks, in contrast to a shorter survival time expected of systemic monocytes. Hayes et al (15) found that microglial cells derived from adult rats rarely expressed NSE, a finding consistent with previous in situ histochemical studies (6).

In vitro properties of human CNS-derived microglia cells isolated from post mortem "normal" adult brain by a rosetting technique, using opsonized human

erythrocytes (EA) to rosette microglia via their Fc receptors, were described by Hayes et al (15). In comparison to blood monocytes, the microglial cells readily became bipolar in culture, did not express NSE, and showed vacuolated cytoplasm. A majority, upon isolation and after seven days in culture, expressed HLA-DR antigens. Proliferation of these cells was described as "non-extensive". Peudenier et al (16) characterized brain-derived macrophages isolated from 8-12 week old human embryos as NSE positive, rarely expressing MHC molecules except following IFN treatment, and as slowly dividing.

We have previously reported that dissociated cell cultures derived from biopsy specimens of adult human brain contain an abundance of cells of the monocyte/macrophage/microglial phenotype (17,18). The purpose of the present study was to address whether the microglial cells derived from surgically resected brain specimens of young adults were distinct from human peripheral blood monocytes under baseline and activating conditions in vitro. Comparisons were also made with adult human astrocytes from the same surgical biopsies. Phenotypic and functional properties of the microglia examined included survival and growth in tissue culture, presence of macrophage/monocyte cell surface molecules and intracellular enzymes, response to IFN treatment in terms of MHC class II antigen expression, proliferation, and the capacity to serve as antigen presenting cells.

MATERIALS AND METHODS

Isolation of microglia

Adult human brain tissue was obtained from surgical resections to ameliorate non-tumor related intractable epilepsy. Ten samples from separate individuals were used for the described studies. Due to the limited number of cells from each sample, different specimens were used for individual experiments. Tissue was obtained from near but not at the epileptic focus. Our method to establish adult human glial cultures have been described in detail (17, 18). To obtain dissociated cells, tissues were treated with 0.25% trypsin and DNAse (50 μg/ml), followed by Percoll (Pharmacia LKB Biotechnology, Uppsala) gradient centrifugation at 15,000 rpm for 30 minutes. Cells

were suspended in culture medium, consisting of Eagle's minimal essential medium (MEM) supplemented with 5% fetal bovine serum (FBS), 0.1% glucose, and 20 μg/ml gentamicin. Two types of cultures were prepared from this initial isolate. In the first, cells were seeded directly onto 9 mm Aclar fluorocarbon coverslips (Dr. S Kim, Vancouver, British Columbia) previously coated with 10 μg/ml poly-l-lysine. These mixed glial cultures contained microglial cells in addition to astrocytes and oligodendrocytes. In the second type of cultures, to obtain enriched microglial cells, the initial cell isolate was plated onto uncoated 25 cm^2 Nunc (Gibco, Burlington, Ontario) flasks. Oligodendrocytes remained floating and were removed the next day. The adherent cells were allowed to develop morphologically for seven days, and residual astrocytes were then floated off by rotary shaking for five hours at 150 rpm. The adherence properties of adult human microglial cells contrast to neonatal rodent preparations where microglial cells are less adherent than astrocytes, and thus can be floated off by rotary shaking. The human microglial cells were detached with 0.25% trypsin and DNAse (50 μg/ml) at 37°C for 15 minutes followed by seeding on 9 mm poly-l-lysine-coated Aclar fluorocarbon coverslips as 30 μl droplets at densities of 10,000 or 25,000 cells per coverslip. The coverslips were adherent to the floor of round tissue culture dishes (60 mm, Nunc). Flooding of culture dishes with 3 ml of culture medium was done one day later. These preparations consistently yielded enriched (greater than 90%) microglia cultures as assessed by Leu-M5 immunofluorescence (see results and Fig. 1).

Isolation of blood monocytes

Peripheral blood monocytes were isolated from heparinized venous blood samples obtained either from the surgical patients or from young volunteers (necessary for flow cytometry studies which required large cell numbers). To isolate monocytes, mononuclear cells obtained by Ficoll-Hypaque (Pharmacia) density centrifugation were incubated overnight, or up to seven days in some experiments, at 37° C in feeding medium described above. The lymphocytes were then washed off; the remaining adherent monocytes were removed with 0.25% trypsin and DNAse (50 μg/ml). These

cells were either used for flow cytometry studies or they were seeded onto poly-l-lysine coated coverslips at densities of 10,000 or 25,000 cells per coverslip. Cells on coverslips were then flooded one day later.

Comparison of microglia and peripheral blood monocytes:

The following characteristics were examined: a) plating efficiency (attachment) and long-term survival, b) morphology and cell size, and c) phenotypic expression of antigenic and enzymatic markers.

A. Plating efficiency and long-term survival

The following cell preparations were compared a) enriched microglia, initially maintained in flasks with astrocytes for seven days and then trypsinized and replated following removal of astrocytes; b) peripheral blood monocytes, maintained in culture overnight following isolation and then trypsinized and replated; and c) peripheral blood monocytes maintained in culture for seven days (to simulate the microglia conditions), then trypsinized and replated. Plating efficiency and long term survival were determined by seeding either 10,000 or 25,000 microglia or monocytes per 30 μl on a coverslip and then flooding the dish containing coverslips with feeding medium one day after seeding. To obtain the percent of surviving cells, one-half of each coverslip was counted. Morphologic properties were also assessed using phase contrast microscopy.

B. Morphology and cell size:

The shape and process extension capability of cultures were assessed after immunofluorescence labelling with Leu-M5 antibody as described below. Comparisons of relative cell size between monocytes in culture for 24 hours and microglia which were cultured for seven days were made by trypsinizing the cells and analyzing live cells in a FACScan (Beckton Dickinson, San Jose, California) comparing forward scatter histograms.

C. Phenotypic expression:

Microglia isolated after seven days in culture and seeded onto coverslips, and peripheral blood monocytes isolated and seeded onto coverslips after either one or seven days, were assessed by immunocytochemistry using antibodies listed in Table 1, and histochemical staining for NSE as described below. Astrocytes and oligodendrocytes were stained using antibodies to glial fibrillary acidic protein (GFAP) and galactocerebroside (GalC). In all staining protocols, sister-culture coverslips were used as controls with HHG (2% horse serum, 1mM Hepes, and 10% goat serum in Hanks balanced salt solution) substituting for the primary antiserum. HHG was the diluting medium of all antibodies. Except for fixation at -20°C, all procedures were done at room temperature.

For immunofluorescence labelling of surface markers, coverslips with adherent cells were washed gently with phosphate-buffered saline (PBS) pH 7.2 (three times) to rinse off the feeding medium. Primary antibody was added for 45 minutes, followed by three PBS washes. Secondary antibody was then added for another 45 minutes. After three PBS rinses, cultures were fixed in acid-alcohol (5% glacial acetic acid: 95% absolute ethanol, v/v) at -20°C for 15 minutes and mounted with gelvatol on microscope slides. For staining of cytoplasmic markers (GFAP, EBM11), cells were first fixed with acid alcohol at -20°C for 15 minutes, washed three times with PBS and stained as described above. For ricinus communis agglutinin-1 (RCA-1), cells were fixed in 2% paraformaldehyde at 4°C for 15 minutes, rinsed three times in PBS and incubated with RCA-1 conjugated to fluorescein isothiocyanate (FITC) for 45 minutes. Non specific esterase was detected using an alpha-napthyl butyrate esterase kit (Sigma #180-B, St. Louis, MO) Briefly, cells on coverslips were fixed at 4°C in a mixture of 2% glutaraldehyde/60% acetone for 5 minutes, rinsed with distilled water and air dried for 15 minutes. A mixture of pararosaniline solution (Sigma #180-4) sodium nitrate, and alpha-naphyl butyrate (Sigma #180-1) was then employed for 1 hour at 37°C. Cells were then rinsed with distilled water, air dried, and mounted.

Assessment of microglial activation

A. MHC Class II expression on microglia

We quantitated MHC class II antigen expression by determining the mean fluorescence intensity (MFI) of anti-MHC class II labelled cells using cytofluorometry. For these studies adherent cells (i.e. microglia), seven days after the initial biopsy/isolation procedure, were trypsinized, suspended, and immunolabelled with a pan MHC class II antibody (SG 465), followed by goat anti-mouse IgG-FITC (45 minutes per antibody at 4°C with three rinses between antibodies). Cells analyzed were either live (for cell size comparisons), or fixed (after staining for MHC class II antigen) with 1% paraformaldehyde in PBS. Five to ten thousand events were accumulated from each sample. Analysis of MFI values were done using the BDIS Consort 30 computer program. Staining controls consisted of unstained cells incubated in HHG, cells incubated with HHG and secondary antibody, and isotype controls incubated with secondary antibody. In some studies recombinant human IFN (100 U/ml) (Boehringer Mannheim, Mannheim) was added to cultures 18 hours prior to preparing cells for cytofluorometry.

Blood monocytes to be analyzed by FACScan were harvested after one day of culture using the same trypsinization protocol and immunostained as described for microglia. Initial studies indicated that there was no significant difference in MFI between cells treated with trypsin and those detached with EDTA or cells that were scraped (data not shown).

To compare HLA-DR expression on microglia to astrocytes, seven day old mixed microglia-astrocyte (GFAP/Leu-M5 positive, GalC negative) cultures were seeded directly onto plastic coverslips following removal from flasks, and double stained with monoclonal antibody D1-12 and anti-GFAP antibody. Using an immunofluorescence microscope, the number of microglia (GFAP negative cells with microglia morphology) or astrocytes (GFAP positive cells) presenting with HLA-DR immunoreactivity were counted. Microglia (Leu-M5 positive) and astrocytes (GFAP positive) accounted for nearly all the cells present in these cultures when oligodendrocytes were removed following initial cell isolation.

B. Functional MHC expression

Functional MHC expression was assayed by the ability of microglia and peripheral blood monocytes to present Candida albicans antigen to freshly derived autologous T-lymphocytes. For these studies, microglia cultured for seven days and peripheral blood monocytes in culture for one day were trypsinized, irradiated (2000 Rad, AECL Gammacell 1000 irradiator, Radiochemical Company, Kanata, Ontario) and plated in 96 well plates at concentrations of 10^3, $5x10^3$, and 10^4 cells per 100 μL of culture medium per well. Culture medium consisted of RPMI 1640 (Gibco, Grand Island, New York) supplemented with 2.5 μg/ml penicillin, 2.5 μg/ml streptomycin, 2 mM glutamine, and 10% FBS. Cells were allowed to adhere overnight either with or without IFN (100 U/ml). The following day T lymphocytes were isolated from peripheral blood by rosetting with neuraminidase treated sheep erythrocytes (Frappier Diagnostic, Laval, Quebec), and suspended at 10^6 cells per ml in 100 μl of culture medium. T lymphocytes were added to microglial/macrophage containing microwells. Candida albicans antigen, 1:20,000 dilution of stock solution (50 protein units/ml, a gift from Dr. CK Osterland, Rheumatology, Royal Victoria Hospital, Montreal, Quebec) was added to some wells. After seven days, cells were pulsed with 1μCi [^{3}H]-thymidine (ICN Biomedicals, Missississauga, Ontario) per well, for five hours and harvested onto glass filters with a Titertek Cell Harvester 550 (Flow Laboratories, ICN, Mississauga, Ontario). Radioactive counts were measured on a LKB 1217 Rack Beta Liquid Scintillation counter (LKB, Fisher, Montreal, Quebec) and were expressed as counts per minute (CPM).

C. Microglia proliferation

To assess microglial proliferation, a Leu-M5/bromodeoxyuridine (BrdU) double immunofluorescence technique, modified from a similar protocol (anti-GFAP/BrdU) for astrocytes (19) was employed. Enriched microglia sister cultures were assessed either under basal culture conditions or after addition for four days of activating agents, listed in the results section. Ten μM BrdU was added during the last two days of culture. At the end of the experiment, live cells on coverslips were labelled with

Leu-M5 (IgG2b isotype) as described above except that the secondary antibody employed was sheep anti-mouse IgG2b conjugated to rhodamine (1:25) (Serotec, Toronto, Ontario). Following fixation with 70% ethanol at -20° C for 30 minutes, cultures were treated successively with 2M HCl and 0.1M sodium borate (10 minutes each), mouse monoclonal antibody to BrdU (IgG1 isotype, Beckton-Dickinson, 1:25, 45 minutes) and sheep anti-mouse IgG1 conjugated to flourescein (Serotec, 1:25, 45 minutes). Using an immunofluorescent microscope, the percentage of Leu-M5 and BrdU double positive cells was tabulated for each coverslip. Assessment of astrocyte proliferation by a GFAP-BrdU technique was done as described previously (19,20).

RESULTS

Comparison of microglia and peripheral blood monocytes:

A. Plating efficiency and long-term survival:

Table 2 shows that microglia seeded at 10,000 cells per 9 mm diameter coverslip had calculated plating efficiencies of greater than 60% when flooded one day after retrypsinization and seeding. At a higher seeding density (25,000 cells per coverslip), overcrowding of microglia prevented reliable counting. In contrast, monocytes flooded at day one after seeding had calculated mean plating efficiencies of 24% and 35%, when seeding densities were 10,000 and 25,000 cells per coverslip, respectively.

Both cell types exhibited a decline in viable cell numbers over time, although microglia had better long-term survival overall (Table 2).

B. Morphology and cell size:

Bipolar cell morphology as illustrated in Figure 1a and b accounted for the majority (80-85%) of the enriched microglial population (90-95% purity as assessed using Leu-M5 immunostaining and FACscan analysis, Figure 1c) present at seven days post-isolation. The remaining cells were either unipolar, with only one process extended, or less frequently ameboid-rounded cells with grainy cytoplasm or ramified

cells (Fig. 2a,c). These cell-shapes were evident by three days following initial cell isolation when Leu-M5 staining could first be appreciated reliably.

The majority of monocytes after seven days in culture were round, phase-bright cells. Between three to seven days in vitro, a small population of process bearing cells (of unipolar or bipolar morphology) could be identified in some, but not all, of the cultures initiated (Fig. 2b,d) Long-term cultures of peripheral blood monocytes (greater than 14 days) had marked debris, with phase dark cells with flattened profiles and short, thick processes.

Microglia cells subjected to Ficoll-Hypaque gradient centrifugation to mimic the isolation procedure of monocytes did not differ in morphological characteristics from sister cultures isolated by the method described for brain cells. Flow cytometric determinations of cell size by forward scatter analysis revealed that the microglial cells which had been in culture for seven days were larger cells than monocytes which had been in culture for 24 hours (Fig. 3).

C. Phenotypic expression

Table 3 summarizes the results of immunolabelling of seven day microglial cultures and monocytes cultured for one or seven days. Both cell types were positive for Leu-M5, EBM 11, MHC class I and II antigens, Fc receptors I and II and RCA-1, and were negative for GFAP and GalC. Peripheral blood monocytes were positive for the NSE reaction (Fig. 4b) and for Leu-M3 and CD4 immunofluorescence at both two and seven days. In contrast to the monocytes, microglia were negative for the NSE (Fig.4a) reaction, and for immunofluorescence staining with Leu-M3 antibody and for the CD4 antigen. Microglia were strongly Fc receptor III immunoreactive while weak immunoreactivity was detected on monocytes.

Assessment of microglial activation

A. MHC Class II expression:

Figure 5 represents microglia and peripheral blood monocytes either unstained or immunostained with anti-MHC class II antibody under basal and IFN stimulated

culture conditions. Using the same cytometer settings between samples, the MFI of unstained or isotype controls for microglia were greater than the MFI of peripheral blood monocytes indicating that the former had a higher level of autofluorescence. The MFI of immunostained monocytes relative to non-stained or isotype control stained monocytes was greater than that for immunostained microglial cells compared to their controls. Interferon gamma consistently increased MHC class II antigen expression on the microglial cells.

Assessment by conventional immunofluorescence microscopy of the number of cells expressing MHC class II antigens showed that under both basal and IFN activating conditions, more microglial cells expressed HLA-DR molecules than did astrocytes (Fig. 6). In a quantitative assessment of mixed glial cultures under basal conditions, 12% of the total astrocytes were positive for HLA-DR expression; in sister IFN stimulated cultures, 56% of the astrocytes were positive. In contrast, basal cultures had 77% positive microglia for HLA-DR while in the IFN stimulated cultures 96% of cells were positive. Ameboid, bipolar, and ramified microglial cells all expressed HLA-DR antigens (Fig. 7).

Additionally, we found that basal levels of HLA-DR expression on microglia in purified cultures increased as a function of time in culture. In a quantitative assay we found that at one week post-purification and seeding on coverslips 63% of the total microglia population were HLA-DR antigen positive, at three weeks post-seeding 84% of the cells were positive, and at five weeks post-seeding 96% of the cells were positive. In contrast, we have previously reported that the percent of astrocytes that express HLA-DR antigens remained constant in culture (18).

B. Functional MHC

As shown in Figure 8, T cells underwent active proliferation in response to Candida albicans antigen in the presence of 1-10% autologous IFN-treated or non-treated microglia. The extent of T cell proliferation was dependent on the proportion of microglial cells present in both unstimulated and IFN stimulated cultures. Proliferation of T cells was greater in the presence of IFN-treated than non-treated microglia at all T cell: microglia ratios tested.

C. Proliferation:

Microglial cells, under basal conditions or in response to IFN (100 U/ml), did not show incorporation of BrdU (Figure 9). None of the following agents tested induced proliferation of microglia: recombinant human (rh) colony stimulating factor-1 (100 U/ml) (Genzyme, Boston, MA), lipopolysaccharide (5 μg/ml) (Calbiochem, La Jolla, CA), human r-interleukin-1 (100 U/ml) (Genzyme), and human r-tumor necrosis factor-alpha (100 U/ml) (Genzyme). Other agents tested that did not cause BrdU incorporation of microglial cells included fibroblast growth factor (20 ng/ml) (UBI, Lake Placid, NY), epidermal growth factor (100 ng/ml) (Collaborative Research Incorporation, Bedford, MA), insulin-like growth factor-1 (20 ng/ml) (Collaborative Research Incorporation), platelet derived growth factor (200 mU/ml) (Genzyme) and nerve growth factor (100 ng/ml) (Sigma, St. Louis, MO). In some experiments, endotoxin free horse serum (a gift from Dr. S Fedoroff, Saskatoon, Saskatchewan) was substituted for FBS, and again no microglia proliferation was observed.

DISCUSSION

The availability of surgical brain biopsy specimens has presented us with an opportunity to study the biological properties of adult human microglia in culture. We have found that microglia and peripheral blood monocytes differ with respect to their physical characteristics in tissue culture including plating efficiency, rate of morphological development, morphology, cell size, survival in culture, antigenic and enzymatic properties, and basal levels of MHC Class II antigen expression. Although we cannot exclude that our "microglia" preparations include some cells from extrparenchymal sources, our data suggest that the majority of microglia in our culture system are indeed cells intrinsic to the brain and not merely blood monocytes trapped in the CNS vasculature removed concurrently during resection.

Our comparative study demonstrates that microglia and peripheral blood monocytes are similar with respect to many immunohistochemical markers of cells from the mononuclear/phagocytic lineage, yet distinct from each other with respect to NSE, CD4, and Leu-M3 where microglia are negative and peripheral blood monocytes

are positive. The observed immunostaining profile of microglia is similar to previous reports that adult human microglia did not express NSE or CD4 (15), and with reports that associated NSE and Leu-M3 with monocytes/macrophages but not microglia (21). Low expression of CD4 mRNA has been detected in microglia with a highly sensitive polymerase chain reaction (PCR) protocol (22). Peudenier et al (16) reported CD4 expression on fetal microglia in culture; as previously mentioned these cells were also NSE positive. Perry and Gordon (23), and more recently Sedgwick et al (24) have also shown CD4 expression on microglia of neonate and three to four week old rats respectively. While the data on CD4 expression in the rat might be explained by species differences (25), the expression on fetal human microglia is more difficult to resolve with the lack of CD4 antigen on adult human microglia. However, the time at which these "microglia" cells in the fetal brain appear is controversial (21, 26, 27) making it difficult to discern between monocytes and resident microglia at this stage developmentally.

Our analysis of MHC class II antigen expression on human adult microglia, in agreement with Hayes et al (15), indicates that the majority of cells express these molecules under basal culture conditions. These results contrast with fetal human and neonatal rat and mouse microglia where expression is observed only after activation with agents such as IFN. Additionally, we found that ameboid, ameboid bipolar, and ramified microglia all can express Class II antigens. Thus, the classification of activation of microglia by the expression of HLA-DR does not fit the idea that ameboid cells are "activated" and ramified are "quiescent" (28). The levels of MHC class II expression on adult microglia under both basal and IFN activating conditions exceeded that observed on astrocytes: these observations parallel those of Sasaki et al (29) using newborn rat CNS cultures. The proportion of MHC class II expressing microglia continued to increase as a function of time <u>in vitro</u> compared to stable values previously observed for astrocytes (18). Our data demonstrate that adult human microglia can present antigen <u>in vitro</u> without the requirement for previous <u>in vitro</u> activation of T cells. This is in contrast to data derived using new born murine astrocytes which only stimulate previously <u>in vitro</u> activated T cells (30) or myelin

basic protein reactive T cell lines (31), indicating that prior T cell activation is a requirement for antigen presentation by astrocytes (30). The Candida albicans antigen used in our study may be acting as a recall antigen rather than serving to prime previously naive T cells.

Results of the proliferation studies point to the inability of adult human microglia to undergo mitosis under basal culture conditions. Similarly, we were unable to demonstrate proliferation of microglia using endotoxin-free horse serum supplemented culture conditions (32) or to demonstrate microglial proliferation in response to the various growth factors. We do observe proliferation of adult human astrocytes under similar culture conditions (20). Several reports have shown that rodent microglial cells could be induced to proliferate when treated with colony-stimulating factors (14, 33, 34, 35). In the present experiment, recombinant human macrophage colony stimulating factor (M-CSF) at 50 or 100 colony forming units (CFU) per ml did not promote proliferation of adult human microglia; it remains undefined whether or not adult human microglia can undergo mitosis under any conditions, or whether these microglial cells represent terminally differentiated cells of the monocyte lineage which are unable to replicate in culture (36, 37, 38). Our current data raise the issue as to whether the human microglial proliferation referred to in vivo under pathologic conditions represents actual proliferation or cell recruitment.

In conclusion we have found that while human adult microglia do share characteristics with peripheral blood monocytes, they differ with respect to adhesion properties and survival in vitro. Microglia, similar to resident macrophages in different tissues express some but not all of the known monocyte/macrophage markers, suggesting that brain microglia/macrophages lose early monocyte markers upon becoming resident brain cells, and that expression of surface and enzymatic markers can be modulated with respect to unique brain microenviroments (28, 39). Based on morphology in tissue culture, we have found three different shapes corresponding to ameboid, ameboid-bipolar, and ramified cells. Microglia of all morphologic subtypes express MHC class II antigens under basal conditions and respond to activation (IFN)

by upregulation of such expression but not by proliferation. Microglia, even under basal conditions, are competent as antigen presenting cells.

REFERENCES

1. Ling EA, Penney D, Leblond CP. Use of carbon labelling to demonstrate the role of blood monocytes as precursors of the "ameboid cells" present in the corpus callosum of postnatal rats. J Comp Neurol 1980;193:631-57

2. Ling EA. The origin and nature of microglia. In: Fedoroff S, and Hertz L, eds. Advances in cellular neurobiology, Vol 2. New York: Academic Press, 1981:33-82

3. Hickey WF, Kimura H. Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vivo. Science 1988;239:290-2

4. Chugani DC, Kedersha NL, Rome LH. Vault immunofluorescence in the brain: new insights regarding the origin of microglia. J Neurosci 1991;11:256-68

5. Oehmichen M. Functional properties of microglia. In: Smith WT, Cavanagh JB, eds. Recent advances in neuropathology, Vol 2. New York: Churchill Livingstone, 1982, 83-107

6. Schelper RL, Adrian EK. Non-specific esterase activity in reactive cells in injured nervous system tissue labelled with ^{3}[H]-Thymidine or I^{125}Iododeoxyuridine injected before injury. J Comp Neurol 1980;194:829-44

7. Kitamura T, Miyake T, Fujita S. Genesis of resting microglia in the grey matter of mouse hippocampus. J Comp Neurol 1984;226:421-33

8. Graeber MB, Streit WJ, Kreutzberg GW. Axotomy of the rat facial nerve leads to increased CR3 complement receptor expression by activated microglial cells. J Neurosci Res 1988;21:18-24

9. Frei K, Siepl C, Groscurth P, Bodmer S, Schwerdel C, Fontana A. Antigen presentation and tumor cytoxicity by interferon gamma treated microglial cells. Eur J Immunol 1987;17:1271-8

10. Woodroofe MN, Sarna GS, Wadha M, Hayes GM, Loughlin AJ, Tinker A, Cuzner ML. Detection of interleukin-1 and interleukin-6 in adult rat brain, following mechanical injury, by in vivo microdialysis: Evidence of a role for microglia in cytokine production. J Neuroimmunol 1991;33:227-36

11. Selmaj KW, Raine CS. Tumor necrosis factor mediates myelin and oligodendrocyte damage in vitro. Ann Neurol 1988;23:339-46

12. Selmaj K, Raine CS, Farooq M, Norton WT, Brosnan CF. Cytokine cytotoxicity against oligodendrocytes, apoptosis induced by lymphotoxin. J Immunol 1991;147:1522-9

13. Giulian D. Ameboid microglia as effectors of inflammation in the central nervous system. J Neurosci Res 1987;18:155-71

14. Suzumura A, Sawada M, Yamamoto H, Marunouchi T. Effects of colony stimulating factors on isolated microglia in vitro. J Neuroimmunol 1990;30:111-20

15. Hayes GM, Woodroofe MN, Cuzner ML. Characterisation of microglia isolated from adult human and rat brain. J Neuroimmunol 1988;19:177-89

16. Peudenier S, Hery C, Montagnier L, Tardieu M. Human microglial cells: characterization in cerebral tissue and in primary culture, and study of their susceptibility to HIV-1 infection. Ann Neurol 1991;29:152-61

17. Yong VW, Antel JP. Culture of glial cells from human brain biopsies. In: Fedoroff S, ed. Protocols for neural cell culture. St. Louis: Humana Press (in press)

18. Yong VW, Yong FP, Ruijs TCG, Antel JP, Kim SU. Expression and modulation of HLA-DR on cultured human adult astrocytes. J Neuropathol Exp Neurol 1991;50:16-28

19. Yong VW, Kim SU. A new double labelling immunofluorescence technique for the determination of proliferation of human astrocytes in culture. J Neurosci Meth 1987; 21: 9-16

20. Yong VW, Moumdjian R, Yong FP, Ruijs TCG, Freedman MS, Cashman N, Antel J. Gamma interferon promotes proliferation of adult human astrocytes in vitro and reactive gliosis in the adult mouse brain in vivo. Proc Natl Acad Sci USA 1991;88:7016-20

21. Esiri MM, Izzi MSA, Reading MC. Macrophages, microglial cells, and HLA-DR antigens in fetal and infant brain. J Clin Pathol 1991;44:102-6

22. Jordon CA, Watkins BA, Kufta C, Dubois-Dalcq M. Infection of brain microglial cells by human immunodeficiency virus type 1 is CD4 dependent. J Virol 1991;65:736-42

23. Perry VH, Gordon S. Macrophages and microglia in the nervous system. Trends Neurosci 1988;11:273-7

24. Sedgwick JD, Schwender S, Imrich H et al. Isolation and direct characterization of resident microglial cells from the normal and inflamed central nervous system. Proc Natl Acad Sci USA 1991;88:7438-42

25. Crocker PR, Jeffries WA, Clark SJ, Chung LP, Gordon S. Species heterogeneity in macrophage expression of the CD4 antigen. J Exp Med 1987;166:613-18

26. Gould SJ, Howard S. An immunohistochemical study of macrophages in the human fetal brain. Neuropathol Appl Neurobiol 1991;17:383-90

27. Choi BH. Hematogenous cells in the central nervous system of developing human embryos and fetuses. J Comp Neurol 1981;196:683-94

28. Dickson DW, Mattiace LA, Kure K, Hutchins K, Lyman WD, Brosnan CF. Microglia in human disease, with an emphasis on acquired immune deficiency syndrome. Lab Invest 1991;64:135-56

29. Sasaki A, Levison SW, Ting JPY. Comparison and quantitation of Ia antigen expression on cultured macroglia and ameboid microglia fro Lewis rat cerebral cortex: analyses and implications. J Neuroimmunol 1989;25:63-74

30. Sedgwick JD, MoBner R, Schwender S, ter Meulen V. Major histocompatibility complex-expressing nonhematopoietic astroglial cells prime only CD8+ T lymphocytes: Astroglial cells as perpetuators but not initiators of CD4+ T cell responses in the central nervous system. J Exp Med 1991;173:1235-46

31. Fontana A, Fierz W, Wekerle H. Astrocytes present myelin basic protein to encephalitogenic T-cell lines. Nature 1984;307:273-6

32. Gebicke-Haerter PJ, Bauer J, Schobert A, Northoff H Lipopolysaccharide-free conditions in primary astrocyte cultures allow growth and isolation of microglial cells. J Neurosci 1989;9:183-94

33. Sawada M, Suzumura A, Yamamoto H, Marunouchi T. Activation and proliferation of the isolated microglia by colony stimulating factor-1 and possible involvement of protein kinase C. Brain Res 1990; 509: 119-24

34. Hao C, Guilbert LJ, Fedoroff S. Production of colony stimulating factor-1 (CSF-1) by mouse astroglia in vitro. J Neurosci Res 1990; 27: 314-23

35. Alliot F, Lecain E, Grima B, Pessal B. Microglial progenitors with a high proliferative potential in the embryonic and adult mouse brain. Proc Natl Acad Sci USA 1991; 88: 1541-5

36. Zuckerman SH, Ackerman SK, Douglas SD. Long-term human peripheral blood monocyte cultures: establishment, metabolism and morphology of primary human monocyte-macrophage cell cultures. Immunology 1979;38:401-11

37. Becker S, Warren MK, Haskill S. Colony-stimulating factor-induced monocyte survival and differentiation into macrophages in serum-free cultures. J Immunol 1987; 139:3703-9

38. Clarkson SB, Ory PA. CD16 developmentally regulated IgG Fc receptors on cultured human monocytes. J Exp Med 1988;167:408-20

39. Perry VH, Gordon S. Resident macrophages of the central nervous system: Modulation of phenotype in relation to a specialized microenviroment. In: Goetzl EJ, Spector NH, eds. Neuroimmune networks: Physiology and disease. New York: Alan P. Liss, 1989:119-25

40. Lanier LL, Arnaout MA, Schwarting R, Warner NL, Ross GD. p150/95 Third member of the LFA-1/CR3 polypeptide family identified by anti-Leu-M5 monoclonal antibody. Eur J Immunol 1985;15:713-8

41. Carrel S, Tosi R, Gross N, Tanigaki N, Carmagnola AL, Accola RS. Subsets of human Ia-like molecules defined by monoclonal antibodies. Mol Immunol 1981;18:403-11

42. Parham P, Barnstable CJ, Bodmer WF. Use of a monoclonal antibody (W6/32) in structural studies of HLA-A, B, C antigens. J Immunol 1979;123:342-9

43. Mannoji H, Yeger H, Becker LE. A specific histochemical marker (lectin Ricinus communis agglutinin-1) for normal human microglia, and application to routine histopathology. Acta Neuropathol 1986;71:341-3

44. Ranscht B, Clapshaw PA, Price J, Noble M, Seifert W. Development of oligodendrocytes and Schwann cells studied with a monoclonal antibody against galactocerebroside. Proc Natl Acad Sci USA 1982;79:2709-13

Table 1: Antibodies used in study

Antibody	Source	Dilution Used	Reference
Leu M5 (mse α-CD11c)	Beckton-Dickinson (San Jose, CA)	1:10	(40)
Leu M3 (mse α-CD14)	Beckton-Dickinson	1:10	Manufacturer
EBM 11	Dako Corporation (Scarborough, ON)	1:50	Manufacturer
Leu 3a (mse α-CD4)	Beckton-Dickinson	1:10	Manufacturer
D1-12 (mse α-HLA-DR)	DR. R Sekaly (Montreal, PQ)	1:1000	
SG465 (mse α-MHC II)	Dr. R Sekaly,	1:1000	(41)
W6/32 (mse α-MHC I)	Dr. R Sekaly,	1:50	(42)
Mab 197 (mse α-CD64, FcRI)	Medarex (West Lebanon, NH)	1:1000	Manufacturer
IV.3 Fab (mse α-CD32, FcRII)	Medarex	1:1000	Manufacturer
3G8 (mse α-CD16, FcRIII)	Medarex	1:1000	Manufacturer
RCA-1	Vector (Burlingame, CA)	1:50	(43)
Mouse α-BrdU	Beckton-Dickinson	1:25	Manufacturer
H8H9 (mse α-GalC)	Dr. S Kim (Vancouver, BC)	neat	(44)
Rabbit α-GFAP	Dako Corporation (West Chester, PA)	1:100	Manufacturer
Goat α-mse Ig-Rh	Cappell (Lexington, MA)	1:150	Manufacturer
Goat α-rabbit-Rh	Cappell	1:100	Manufacturer
Goat α-rabbit-FITC	Cappell	1:100	Manufacturer
NSE (enzyme)	Sigma, Kit #180-B		Manufacturer

Abbreviations used: MSE= mouse, Rb= rabbit, Mtl= Montreal, N.H.=New Hampshire

Table 2: Microglia and peripheral blood monocytes in culture: plating efficiency and cell survival

	Plating Efficiency %	Survival %	
	Day 1	Day 7	Day 14
microglia			
10,000/coverslip	61-71	61-64	51-54
25,000/coverslip	Too densely populated	to count	reliably
peripheral blood monocytes			
10,000/coverslip	22-26	15-29	19-22
25,000/coverslip	33-37	35-41	24-29

Plating efficiency and percent cell survival comparison between microglia (after seven days in culture) and peripheral blood monocytes (after one day in culture). Cells were seeded onto coverslips at 10,000 or 25,000 cells per coverslip (n=3 coverslips per data point), adhered overnight, and flooded. Percents expressed as number of cells remaining on coverslip over original number of cells seeded. Peripheral blood monocytes left in culture for seven days, trypsinized, and seeded at 10,000 cells per coverslip had survival ranges of 18-24% at two days and 3-7% at 14 days.

Table 3: Phenotypic expression on 7 day microglia and 1 and 7 day peripheral blood monocytes

Marker	Microglia	Peripheral Blood Monocytes
Leu M5	++	++
Leu M3	-	++
EBM 11	++	++
α-CD4	-	+*
α-MHC class I	++	++
α-MHC class II	++	++
α-FcRI	++	++
α-FcRII	++	++
α-FcRIII	++	+**
RCA-1	++	++
NSE	-	++
α-GFAP	-	-
α-GalC	-	-

++ easily detectable in the large majority of cells,

\+ weakly stained throughout the given culture,

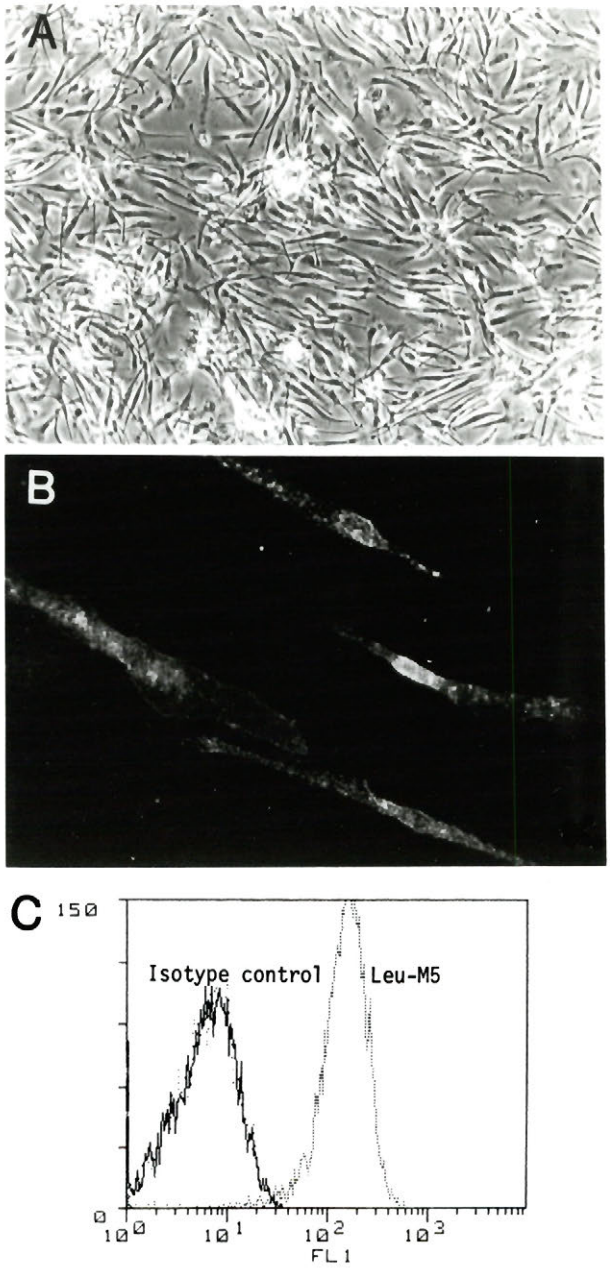
\- undetectable in the large majority, if not all cells, of a given culture

* stain intensity diminishes with time in culture,

** stain intensity increases with time in culture.

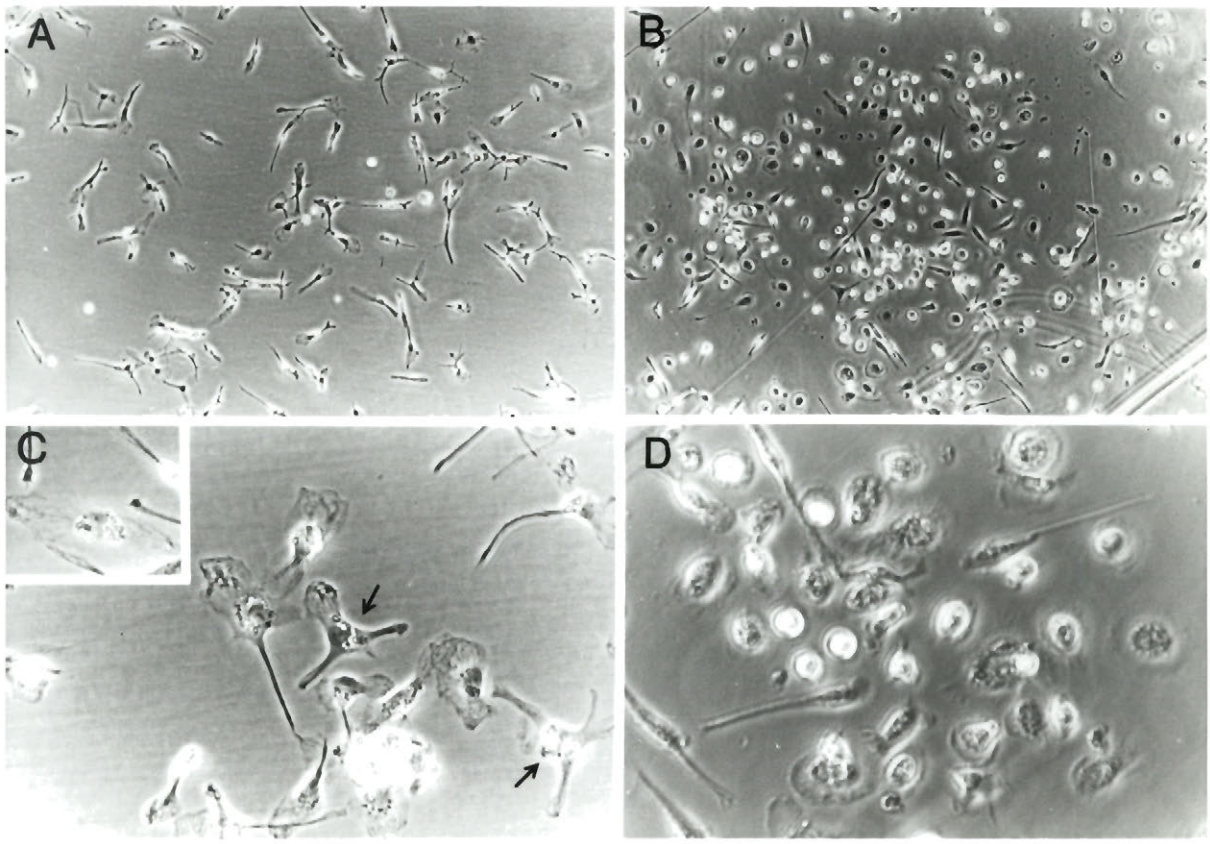
FIGURE LEGENDS

Figure 1. Enriched adult human microglia in culture. A. Microglia cultured for seven days, purified, trypsinized and seeded onto Alcar coverslips at density of 10,000 cells per coverslip (250x). B. Leu-M5 immunoreactivity on seven day microglia cultures (1000x). C. Flow cytometry histogram of seven day purified microglial culture stained with Leu-M5 antibody. Curves represent unstained cells overlapping with isotype control, and Leu-M5 (primary) FITC (secondary) stain. This staining pattern, shown here for one culture, was reproducible among different series.


A
B
C
150
Isotype control
Leu-M5
0
10^0
10^1
10^2
10^3
FL1

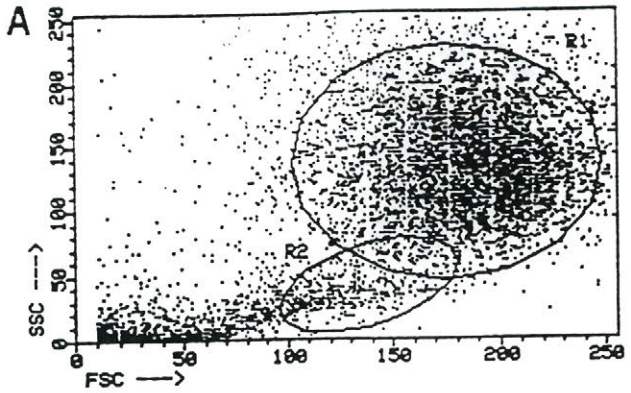
92-0326

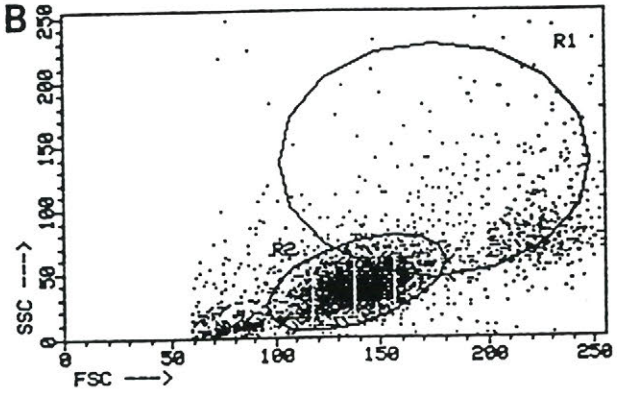
Figure 2. Comparison of microglia and peripheral blood monocytes in culture. A. Enriched, seven day microglia culture (200x). B. Enriched peripheral blood monocytes after seven days in culture (200x) C. Higher magnification (800x) of a seven day microglia culture with ramified (arrows) and ameboid (inset) cells. D. Higher magnification (800x) of a seven day peripheral blood monocyte culture; majority of cells remain rounded with a small population with bipolar morphology.


A
B
C
D

92-0329

Figure 3. Flow cytometric comparisons between microglia and peripheral blood monocytes using the same cytometer amplification and detection settings, and serial sample runs. A. Forward scatter (FSC) plot of microglial cells, seven days in culture. The large oval (R1) represents population gate of cells selected for analysis. B. Forward scatter of peripheral blood monocytes one day in culture, small oval (R2) is population gate (note: in order to analyze both microglial and peripheral blood monocyte populations in the same forward scatter fields cytometer settings are lower than traditionally used for mononuclear cells; thus peripheral blood monocytes cluster lower than is usually seen by dot plot). C. Overlay histogram of forward scatter plots showing microglial cells, the larger cell of the two populations, and peripheral blood monoctyes.


A
R1
R2
SSC ---->
FSC ---->
0
50
100
150
200
250


B
R1
R2
SSC ---->
FSC ---->
0
50
100
150
200
250

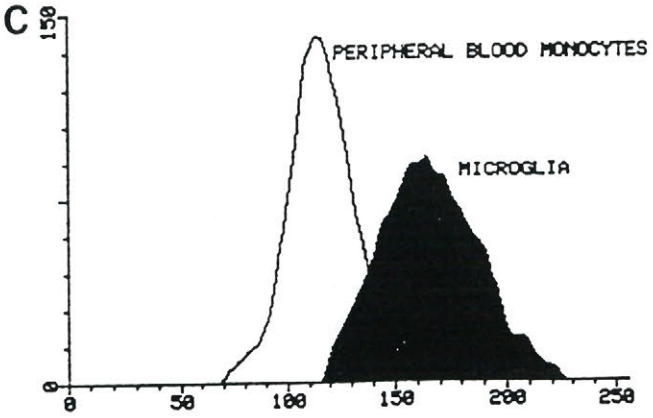
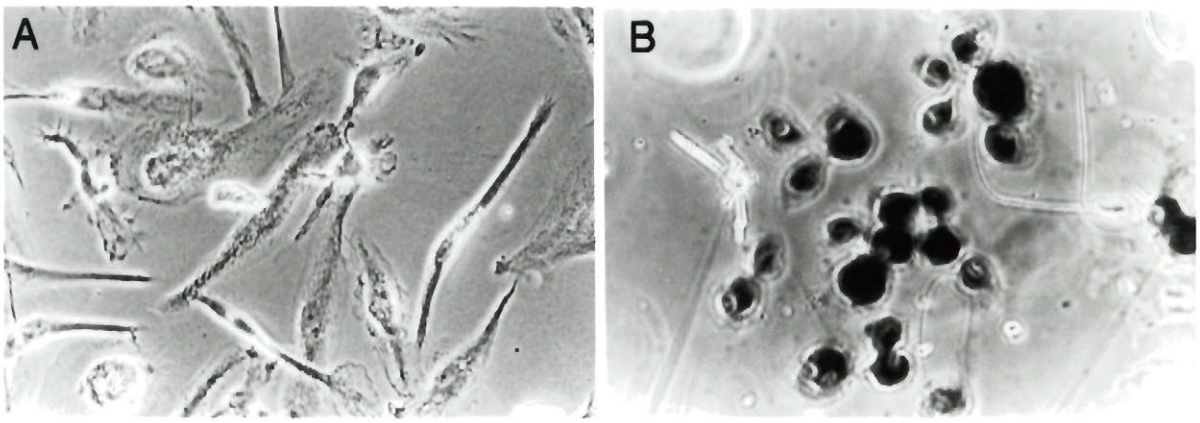
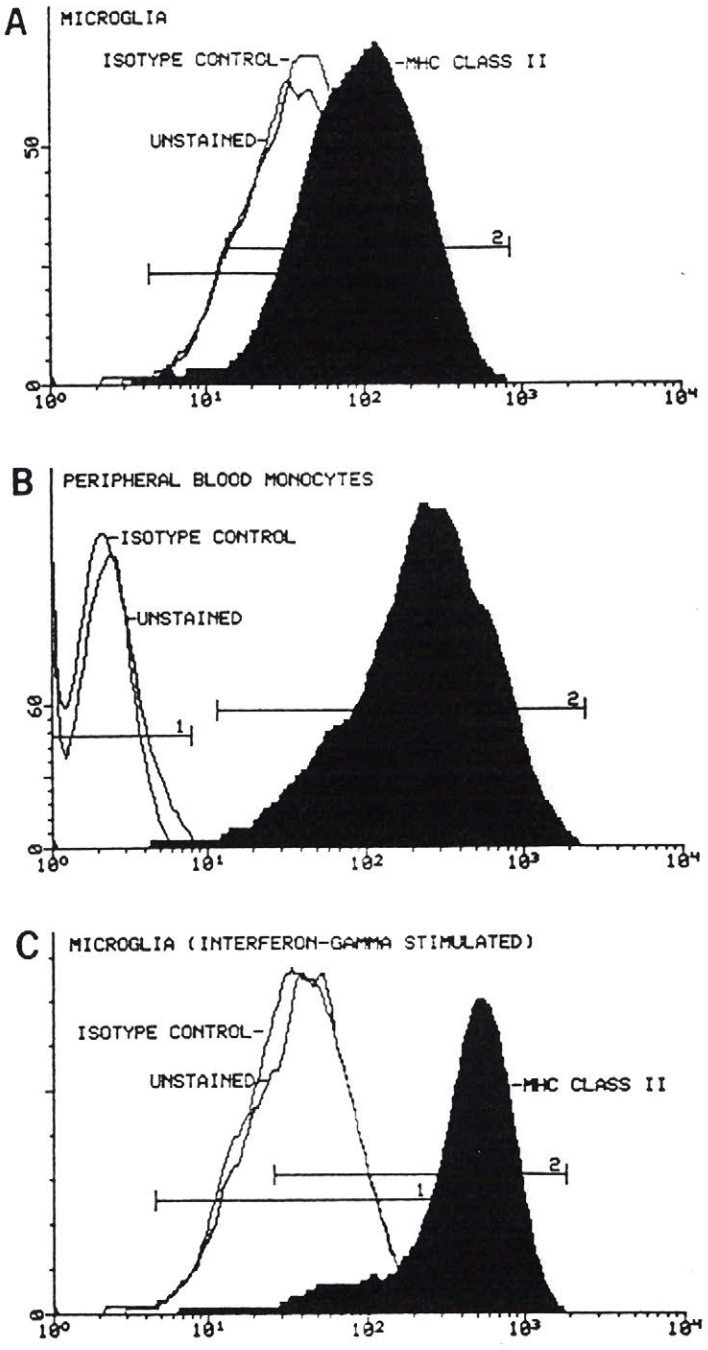

C
PERIPHERAL BLOOD MONOCYTES
MICROGLIA
0
50
100
150
200
250

Figure 4. Non-specific esterase reaction of: A. Microglia in culture for seven days (800x) and B. Peripheral blood monocytes in similar culture conditions (800x).


A
B

92-0327

Figure 5. Flow cytometry of basal and IFN stimulated class II antigen expression. A. seven day purified microglial culture either unstained or immunostained isotype control (MFI=100.91) or with anti-MHC class II (SG465) (MFI=131.07). B. one day peripheral blood monocyte histograms of isotype control (MFI=5.53) or immunostained with SG465 (MFI=333.09). C. MHC Class II antigen expression on seven day microglial cultures with unstained and isotype control (MFI=132.83) and IFN stimulated cells (MFI=492.88). The staining characteristics, shown here for one culture, were reproducible among different human cultures.


A
MICROGLIA
ISOTYPE CONTROL
MHC CLASS II
UNSTAINED
50
0
2
10^0
10^1
10^2
10^3
10^4
B
PERIPHERAL BLOOD MONOCYTES
ISOTYPE CONTROL
UNSTAINED
60
0
1
2
10^0
10^1
10^2
10^3
10^4
C
MICROGLIA (INTERFERON-GAMMA STIMULATED)
ISOTYPE CONTROL
UNSTAINED
MHC CLASS II
0
1
2
10^0
10^1
10^2
10^3
10^4

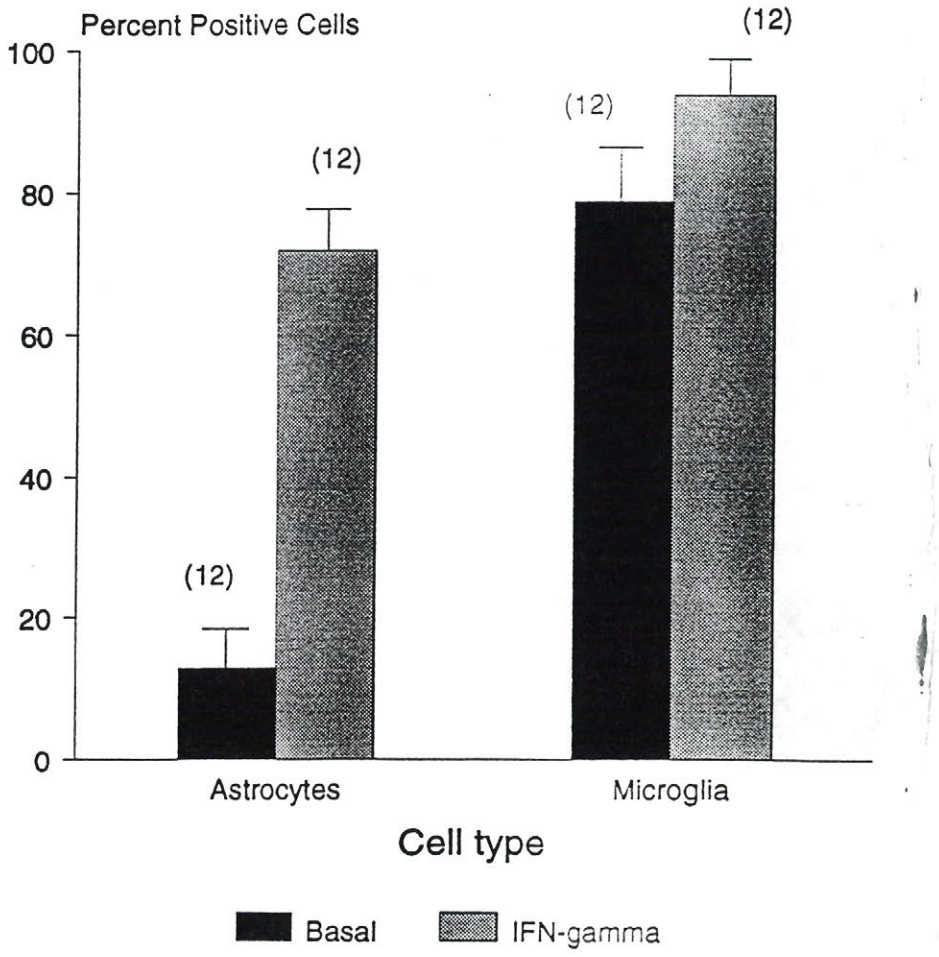
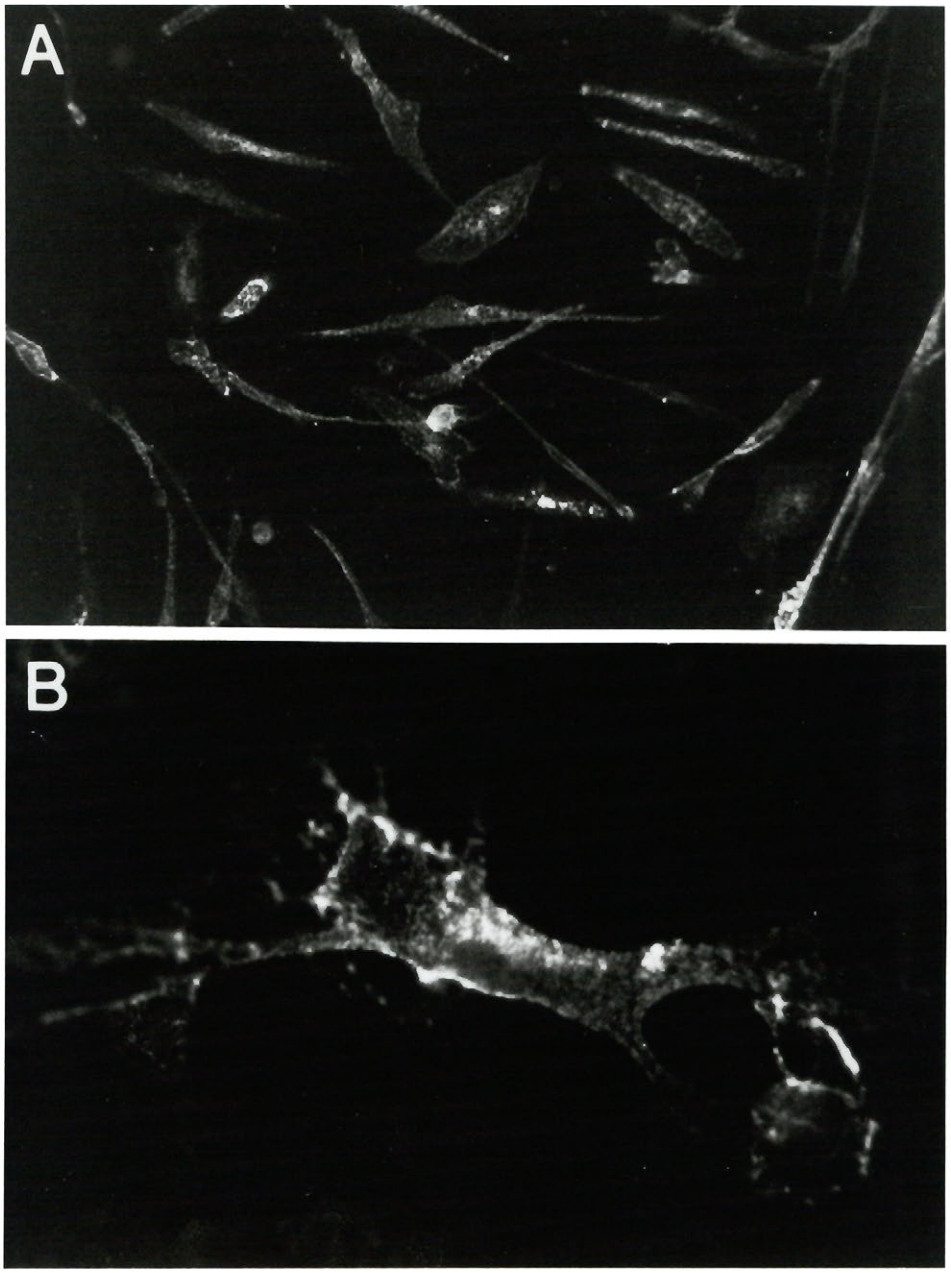


Figure 6. Comparison between basal and IFN (100 U/ml) stimulated HLA-DR (D1-12) staining of adult human astrocytes and microglia in mixed glial cultures. Astrocytes were identified with GFAP antibody. Bars represent the percent of astrocytes or microglial cells that were immunoreactive for HLA-DR $\pm$ SEM. Number of coverslips analysed is shown in parentheses.

Figure 7. Purified seven day microglial cultures stained for HLA-DR. A. Low power magnification (400x) showing immunoreactivity on both bipolar and ameboid cells. B. High power magnification showing HLA-DR reactivity on a ramified microglial cell (1000x).


A
B

92-0329

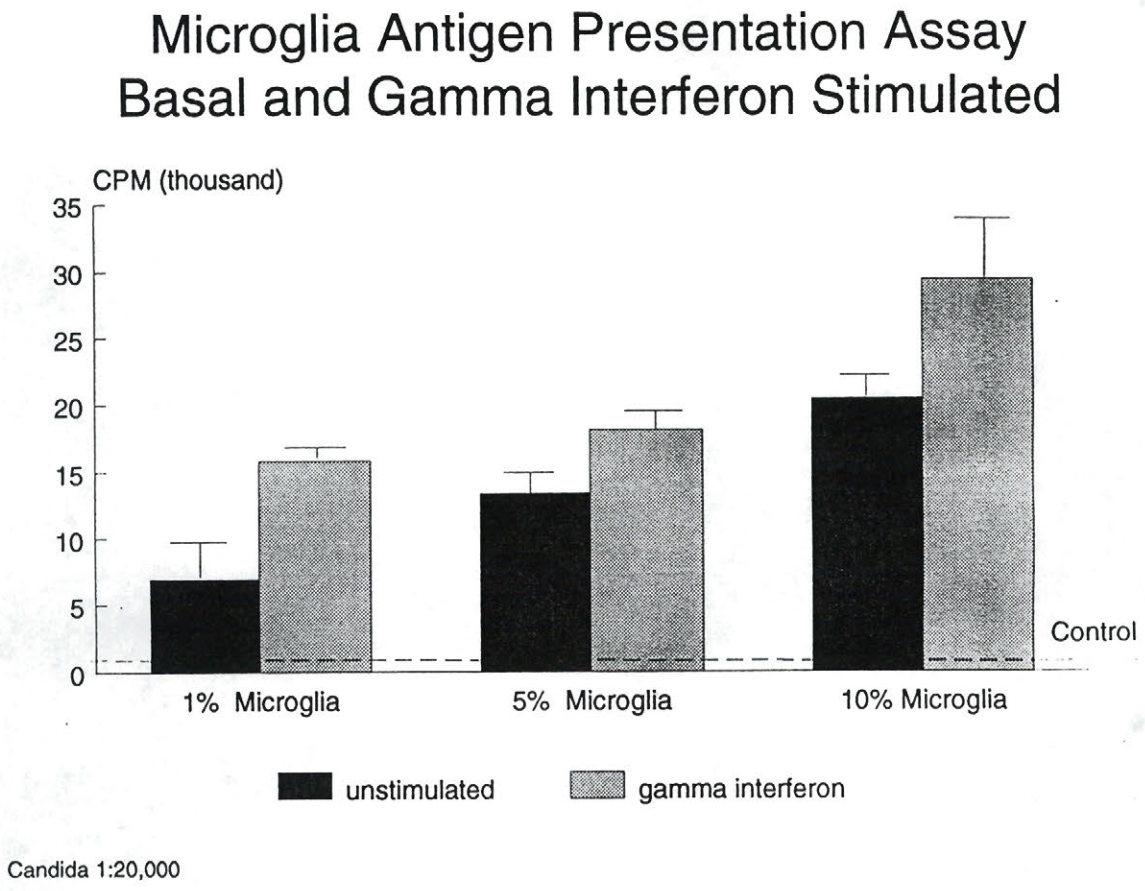
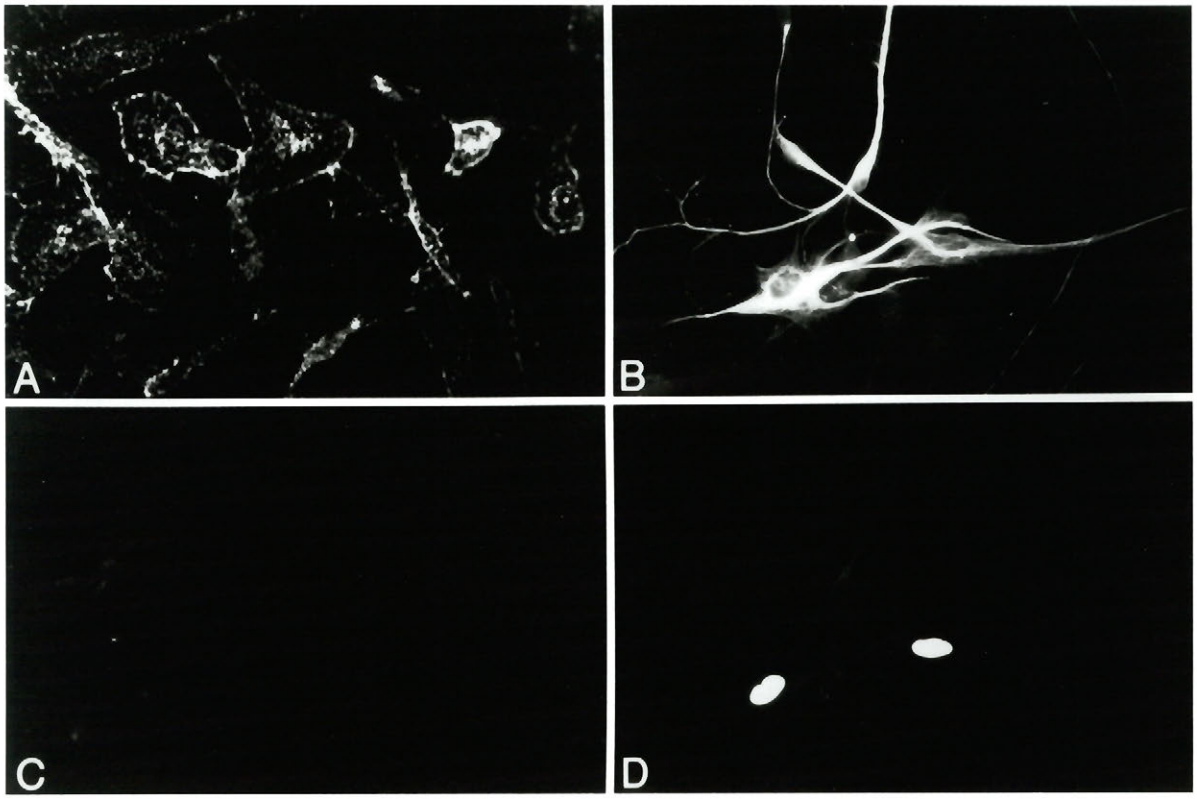


Figure 8. Microglia as antigen presenting cells. Bars represent counts per minute (CPM) of 10^5 T cells cocultured with either 10^3 (1%), $5x10^3$ (5%), or 10^4 (10%), microglial cells plus Candida albicans antigen, ± IFN. Each bar represents the mean ± SEM of four wells. CPM of T cells, T cells plus microglia without antigen, or microglia cells alone were below 500 cpm as represented by control line at the bottom of the graph.

Figure 9. Mixed microglia and astrocyte cultures assessed for proliferation by BrdU incorporation. A. Microglia stained with Leu-M5 antibody (800x). B. Astrocytes stained with anti-GFAP antibody (800x). C. Same microglial field as A, with no reactivity to anti-BrdU antibody (800x). D. Same astrocyte field as B, showing BrdU incorporation (800x).


A
B
C
D

92-0346

Chapter 3

PHENOTYPIC DIFFERENCES BETWEEN HUMAN MONOCYTES/MACROPHAGES AND MICROGLIA IN SITU AND IN VITRO

Elling Ulvestad, Kenneth Williams, Sverre Mørk,
Jack Antel, and Harald Nyland

Preface

From the work described in Chapter 2, using the differential staining patterns of microglia and peripheral blood monocytes in vitro, it was of interest to determine if the same staining phenotype would hold in situ, and if other established markers used on myeloid phagocytic cells might assist in further differentiating between these two cell populations. This chapter describes data investigating the possibility of delineating microglia and monocytes/macrophages infiltrating the CNS in acute and chronic inflammatory disease. Major points demonstrated in the chapter include:

1. A confirmation of earlier in vitro data (Chapter 2) that monocytes infiltrating the CNS were positive for the markers non-specific esterase and Leu-M3 while microglia were negative for both markers.
2. Monocytes recently infiltrating the CNS were also positive for other myeloid markers including myeloperoxidase, L1, lysozyme, and RFD7, and again microglia were negative for the same markers.
3. Using the immune stain pattern described in Chapter 2, it was demonstrated that monocytes invading the CNS in chronic MS lesions have primarily a perivascular location while in active inflammatory lesions, monocytes and macrophages also infiltrate into the parenchyma.

ABSTRACT

This report describes a phenotypic differentiation pattern conceived to distinguish invading monocytes/macrophages from resident microglia in frozen and formalin fixed CNS. Phagocytic cells in normal and diseased CNS (MS and encephalitis) were studied immunohistochemically with a panel of antibodies, and phenotypic characteristics compared with cultured monocytes/macrophages and microglia. Monocytes/macrophages were positive for the markers non specific esterase, myeloperoxidase, L1, lysozyme, RFD7, and CD14, whereas microglia were negative for the same markers. Both populations of cells were positive for CD11c and CD68. Our results indicate that invading monocytes/macrophages mainly have a perivascular location in established lesions of multiple sclerosis brains, whereas invading phagocytes also infiltrate the parenchyma in acute inflammatory CNS diseases, such as in encephalitis.

INTRODUCTION

The origin and phenotypic characteristics of resident microglial cells in the human central nervous system (CNS) has long been a subject of controversy. Microglia were first described by del Rio-Hortega as distinct from astroglia and oligodendroglia (1,2). Since then several hypotheses for the embryonic origin of microglia have been proposed, suggesting that they are derived from pial elements, from neuroectodermal matrix cells, from pericytes, or from brain invasion by blood monocytes (3). Microglial cells express various molecules which have been detected only on cells of bone marrow origin, including CD45 (leukocyte common antigen) and CD64 (IgG Fc receptor I) (4), favoring the concept that microglia are bone marrow-derived.

A distinction has been made between parenchymal microglia and perivascular cells. Perivascular cells are separated from the CNS parenchyma by a basement membrane, whereas microglia are true parenchymal cells (5). Studies using bone marrow

chimeric rats provide strong evidence that perivascular cells are bone marrow-derived and continuously repopulated, whereas no significant repopulation of parenchymal microglia by hematogenous cells occurs, indicating that parenchymal microglial cells are more permanent residents of the CNS (6,7).

Microglia are of importance in the CNS immune response. They express MHC class II molecules constitutively, establishing them as potent intraparenchymal antigen presenting cells (8). In addition, microglia have phagocytic capacity (9). It is currently believed that both invading monocytes/macrophages and resident microglia may contribute to the phagocytic MHC class II expressing cells observed in the injured adult brain. To assess the relative contribution of microglia and monocytes/macrophages to human CNS disease processes, a phenotypic differentiation of the two cell populations is required. However, no antigens specific for microglia have as yet been found, and identification of microglia in vivo and in vitro is therefore performed using markers characterisitic of monocytes/macrophages, making the differentiation between invading monocytes/macrophages and microglia difficult.

In an attempt to further characterize and differentiate the inflammatory phagocytes in CNS disease, we have utilized various markers of monocytes/macrophages. Tissue sections of normal and diseased brains were studied. In addition, a recently developed cell culture technique has enabled us to study purified microglia in vitro. Phenotypic characteristics of cultured microglia were compared with characteristics of cultured monocytes and monocyte-derived macrophages. Our data indicate that microglia can be differentiated from invading monocytes and macrophages in the injured brain, and a phenotypic characterization pattern for microglia as opposed to monocytes/macrophages is proposed.

MATERIALS AND METHODS

Patients

The characteristis of patients with inflammatory CNS diseases are summarized in Table 1. Five patients had clinically definitive MS. Patients 1-3 had remittent-progressive MS, patients 4 and 5 had remittent-relapsing MS. Patients 6-8 died of

acute viral encephalitis, whereas patient 9 died with a chronic Epstein-Barr virus encephalitis. The diagnosis was confirmed by post mortem examination of the CNS. Three patients with non-cerebral diseases were included as controls.

Tissue

Brain and spinal cord from patients and controls were obtained at autopsy. Tissue from four MS patients and three control brains were blocked in Cryomount (Histolab, Gøteborg, Sweden) and frozen in isopentane precooled in liquid nitrogen. Tissue from MS patients and patients with encephalitis were fixed in 10% formalin and embedded in paraffin. Sections were cut (4-10 μm) on a cryostat. Frozen sections were postfixed for 5 min in cold acetone. Histological staining of sections with Luxol fast blue was performed to delineate demyelinated regions, with hematoxylin and eosin (H&E) to reveal mononuclear cells within the lesions, and with Oil red O to identify cells with neutral fat in their cytoplasm. Hypercellular MS lesions with perivascular inflammatory cells and numerous Oil red O positive cells were assessed as active. Temporal lobe tissue was removed from patients undergoing surgical procedures for intractable epilepsy by Cavitron ultrasonic aspiration. The procedure dissected the tissue into multiple pieces 2-3 mm^3 in size, providing the material used to establish CNS cell cultures.

Microglia cultures

Isolated glial cells were obtained by a previously described protocol (10). In brief, CNS temporal lobe tissue from patients undergoing surgical procedures for intractable epilepsy was cut into small pieces and trypsinized. The single cell suspension was then centrifuged over a Percoll gradient (Pharmacia LKB, Uppsala, Sweden). Glial cells were recovered from the layer of floating cells between the top layer of myelin and the bottom layer of red blood cells. Cells were seeded in tissue culture flasks (Nunc, Roskilde, Denmark) at 10 million cells per flask and grown in a humid atmosphere at 37^{0}C with 5% CO_2 for seven days. The less adherent cells were shaken off into the culture medium by rotary motion (150 rpm) for 5 h at room

temperature and removed. The more adherent microglia were finally removed with 0.25% trypsin and seeded on poly-L-lysine coated cover slips at a density of 10.000 cells per cover slip. They were cultivated for up to seven days before analysis. Culture medium was Eagle's minimum essential medium supplemented with 5% FCS, gentamicin 20 μg/ml, glucose 1 mg/ml, and 2 Mm glutamine (all from GIBCO, Burlington, Ont). In some experiments, interferon-γ (IFN-γ) was added at 100 U/ml (UBI, Lake Placid, NY) for 24 - 48 hours before analysis.

Monocytes and monocyte-derived macrophages

Blood donor buffy coats were centrifuged on Isopaque-Ficoll (Lymphoprep, Nyegaard & Co., Oslo, Norway) density gradients (11). Peripheral blood mononuclear cells (PBMC) were recovered from the interphase, and washed three times in phosphate buffered saline, pH 7.2 (PBS). The cell suspension was adjusted to 20 x 10^6 cells/ml in RPMI 1640 medium supplemented with 2 mM glutamine, 20 μg/ml gentamycin and 10% FCS (GIBCO). 0.4 ml PBMC were seeded into the wells of 8 chamber culture slides (Nunc Inc., Naperville, Ill., U.S.A), and cultured at 37^{0}C in a humid atmosphere with 5% CO_2 for one hour. Non-adherent cells were then removed by gentle washings in warm PBS. Medium was added and cultures reincubated. Monocytes were studied immediately after isolation, whereas monocyte-derived macrophages were cultured for 8 days. Medium was changed every second day.

Antibodies

The antibodies used in this study are summarized in Table 2. Isotype control mAbs were MOPC21 (IgG1), UPC10 (IgG2a), and MOPC195 (IgG2b), obtained from Sigma, St. Louis, USA. Isotype controls and all anti-CD14 mAbs were used at 5 μg/ml. Biotinylated rabbit anti-mouse immunoglobulins and HRP-conjugated swine antibodies to rabbit IgG were purchased from Dakopatts, Glostrup, Denmark.

Immunohistochemistry

Endogenous peroxidase was inactivated by pretreatment with 0.3% H_2O_2 for 10 min. Sections to be stained by mAbs were thereafter preincubated with 10% rabbit serum in PBS for 30 min at room temperature, whereas sections to be stained by polyclonal rabbit antibodies were preincubated with 10% human serum. Sections were then incubated over night at 4°C in a humid chamber with mAbs diluted in 10% rabbit serum or with polyclonal rabbit antibodies diluted in 10% human serum.

We have recently shown that microglia in MS lesions strongly express receptors for the Fc portion of IgG (FcR) (12). To investigate the specificity of mAb reactivity, control experiments were therefore performed with or without FcR-blocking. The various anti-CD14 mAbs and isotype controls were diluted in either PBS or in PBS with 10% rabbit serum, and applied to sections preincubated with or without 10% rabbit serum for 2 hours at room temperature.

Biotinylated rabbit anti-mouse immunoglobulins or HRP-conjugated swine antibodies to rabbit IgG were then applied for 30 min. Avidin-biotin-peroxidase complex (ABComplex/HRP, Dakopatts) was prepared as recommended by the manufacturer, and allowed to react with sections for 30 min. Sections were finally treated with a 3-amino-9-ethyl-carbazole containing buffer for the development of a colored reaction product. All incubations were followed by washings in PBS. Sections were finally counterstained with hematoxylin, mounted in Glycergel (Dakopatts), and analyzed in a light microscope.

Viable microglia were stained with mAbs for 60 min at 4°C. The cells were then fixed with 95% ethanol-5% glacial acetic acid at -20°C for 15 min. Microglia were therafter immunostained as described above.

Monocytes and monocyte derived macrophages were pretreated with 0.3% H_2O_2 for 10 min before immunostaining with antibodies.

Non-specific esterase (NSE)

The cytochemical reaction for NSE was performed according to the method described by Yam et al. (13) In brief, cryostat sections of tissue or cultured cells were

fixed for 30 s in phosphate buffered acetone-formaldehyde. Sections were then incubated at room temperature for 45 min in the following solution: 44.5 ml 0.15M phosphate buffer, 50 mg α-naphtyl acetate dissolved in 2.5 ml ethylene glycol monomethyl ether, and 3 ml hexazotized pararosaniline (reagents from Sigma, St. Louis, USA). The solution was adjusted to pH 6.1 with 1N NaOH and filtered. After incubation, sections were washed and counterstained with hematoxylin before mounting.

Endogenous peroxidase

The demonstration of myeloperoxidase activity in cells was done according to the method described by Kaplow (14), with modifications. In brief, cultured cells or tissue sections were fixed for 60 s at room temperature in 10% formal-ethanol, and then washed with water. Cells were thereafter incubated for 60 s in the following solution: 10 mg 3-amino-9-ethyl-carbazole diluted in 6 ml dimethyl sulfoxide and 50 ml 0.02M sodium acetate, pH 5.5. 4 μl 30% H_2O_2 was added immediately before use. Cells were therafter washed in water, counterstained with hematoxylin, and mounted.

RESULTS

Normal white matter

Perivascular cells showed diffuse NSE activity, but no myeloperoxidase activity. Cells in the parenchyma expressed neither NSE nor myeloperoxidase activity. MAC 387 and anti-lysozyme reacted with occasional cells within the vessels. Neither perivascular cells nor cells in the parenchyma were stained with MAC 387 and anti-lysozyme. RFD7 and anti-CD14 mAbs stained perivascular cells (Fig 1A-D). MY4 gave the strongest staining intensity. No parenchymal microglia were stained by anti-CD14 mAbs. Both perivascular cells and parenchymal cells were stained by mAbs to CD11c, CD45, CD64, and CD68 (Fig 1E; Table 3). CD68 reactivity appeared to be intracellular and granular. Isotype controls did not bind to normal CNS.

MS lesions

In H&E stained sections a perivascular mononuclear infiltrate was observed within the lesions. Luxol fast blue delineated the demyelinated regions, and lipid filled cells were stained by Oil red O.

Diffuse cellular NSE reactivity was found on cells in the perivascular spaces. A few scattered cells in the parenchyma were also NSE positive (Fig 1F). Myeloperoxidase activity was observed on occasional mononuclear cells in the perivascular spaces and on cells within the vessels, but not on microglia. MAC 387 and anti-lysozyme stained intravascular polymorphonuclear cells and some mononuclear perivascular cells, but rarely detected any parenchymal cells. In cryostat sections, MAC 387 reacted with antigen diffusely surrounding the vessels, probably due to leakage of the L1 protein. No such leakage was observed in formalin fixed tissue. RFD7 mAb stained perivascular cells only in most of the lesions (Fig 2A,B). In one active lesion (patient 4) a significant infiltration of RFD7 positive cells was evident. These cells were also CD14 positive (Fig 2C)

The four anti-CD14 mAbs displayed heterogenous staining characteristics within some of the lesions. In experiments where rabbit serum was omitted from incubations, TÜK4 and the IgG2a isotype control UPC10 stained a high proportion of the parenchymal cells (Fig 2D). Reactivity with TÜK4 was also observed in some small areas of apparently normal CNS outside the demyelinating lesion. The non-specific binding of IgG2a mAbs was further substantiated by the use of mAbs reactive with CD21. No binding to microglia was detected by the IgG1 anti-CD21 mAb, whereas the IgG2a anti-CD21 mAb reacted with microglia within the lesions. This binding could be completely abrogated by preincubation of sections with 10% rabbit serum. Leu-M3, MY4, and AML 2-23 did not stain cells in the parenchyma in most lesions, whereas perivascular cells were stained (Fig 2E). No cells were stained by the IgG1 isotype control, whereas IgG2b isotype control reacted with occasional cells within the lesions. In experiments with 10% rabbit serum included, perivascular cells remained positive for CD14, whereas most of the parenchymal cells were negative. Isotype controls

were also negative. MY4 stained perivascular cells strongly, and also stained occasional cells within lesions which were not detected by the tree other anti-CD14 mAbs.

Cells reactive with mAbs to CD11c (Fig 2E), CD45, CD64, and CD68 were prominent throughout the lesions. The intensity of staining of all these mAbs was stronger within lesions and at the borders than in the normal appearing surrounding parenchyma. The mAb to CD68 also stained endothelium in some lesions. The mAb reactive with CD45 stained a majority of cells in the perivascular mononuclear cell infiltrates.

Encephalitis

In the CNS from the three patients with acute encephalitis, perivascular mononuclear inflammation and parenchymal hemorrhages were evident in sections stained with H&E. Occasional plasma cells were observed around the vessels. Demyelination was perivascular and not as sharply delineated as in MS lesions. Mononuclear cells reactive with anti-lysozyme, MAC 387, CD45, and CD68 were distributed around vessels and in the parenchyma (Fig 3A-C). More cells were stained by the mAb to CD68 than with MAC 387 and anti-lysozyme.

The H&E staining of the CNS from the patient with a chronic CNS inflammation displayed few perivascular inflammatory cells. No cells reactive with anti-lysozyme or MAC 387 could be demonstrated in the parenchyma, whereas CD68 positive cells were abundant.

Characterization of cultured microglia, monocytes and macrophages.

Cultured microglia were negative for non specific esterase, myeloperoxidase, lysozyme, L1, and RFD7 (Table 3). A majority of microglia were also negative for CD14 and NSE; with occassional cells showing weak reactivity. The expression of these molecules was not induced on microglia after treatment with 100 U/ml IFN-γ. Microglia were positive for CD11c, CD45 (Fig 3D), CD64 and CD68. Monocytes and monocyte-derived macrophages were positive for the same markers as microglia,

but also for non specific esterase, myeloperoxidase (monocytes only), lysozyme, L1, RFD7 (macrophages only), and CD14 (Table 3). Expression of L1 and CD14 was weaker on monocyte derived macrophages than on monocytes.

DISCUSSION

The study of inflammatory CNS diseases has been hampered by the lack of specific markers for microglia. Microglia have therefore been identified using markers also recognizing monocytes/macrophages, making it difficult to distinguish the differential role of these cells in CNS pathology. In the normal human CNS, microglia can be distinguished from monocytes/macrophages by their ramified morphology and by their localization. Morphological and anatomical distinctions between microglia and monocytes/macrophages can however not be relied on in the inflamed CNS, where invasion of hematogenously derived monocytes and lymphocytes may occur. Furthermore, microglia may transform into reactive macrophage-like cells. It has therefore been difficult to ascertain the relative importance of invading monocytes and resident microglia to disease processes within the CNS. This problem is reflected by the various names given to phagocytic cells within MS lesions; macrophages (15), foamy macrophages (16), microglial lipophages (17), and foam cells (18).

Several studies have addressed the problem of identifying hematogenous phagocytic cells within MS lesions, but so far no systematic differentiation between monocytes/macrophages and microglia has been established. In an attempt to identify microglia in inflammatory CNS diseases, we have therefore employed several markers of myeloid phagocytic cells. Both acute and chronic CNS diseases were studied, and results were compared with <u>in vitro</u> studies of brain and blood derived phagocytes. As demonstrated in Table 3, several markers distinguish between microglia and monocytes/macrophages. In frozen sections, invading monocytes/macrophages can be identified by the markers CD14, NSE, MPE, L1, lysozyme, and RFD7. In formalin fixed sections, hematogenously derived cells can be identified by the markers MAC 387 and lysozyme. Resident microglia, being negative for these markers, can be identified by mAbs to CD11c (frozen sections only) and CD68.

Our results show that microglia are the major phagocytic cells within MS lesions. Monocytes were observed mainly in the perivascular inflammatory cuffs. These results are somewhat in contrast to results published by Woodroofe et al. (19) and to studies performed by Esiri and Reading (15). Both groups used the mAbs UCHM1 (CD14, IgG2a) and EBM11 (CD68, IgG1) to identify phagocytic cells in MS lesions. In both studies these markers stained similar numbers of cells; the majority of parenchymal cells reacted with both mAbs. UCHM1 also stained cells with microglial appearance beyond the margins of active lesions. In control studies performed by Esiri and Reading, microglia in the normal CNS were negative for CD14 and positive for CD68, thereby indicating that phagocytic cells in MS lesions are mainly hematogenously derived monocytes. However, no blocking of FcR were performed in these studies, and the binding of UCHM1 to parenchymal cells might therefore have been non-specific due to the high affinity of FcRI for IgG2a mAbs (20). This hypothesis is strengthened by our identification of non specific binding of IgG2a mAbs to parenchymal and border zone cells, and by the identification of enhanced FcRI staining on these cells. Non-specific IgG2a binding to microglia was not evident in normal CNS, and enhanced non-specific IgG2a reactivity could thus be used as a marker of activated microglia. Hofman et al. (21) identified Leu-M3 positive cells mainly at the lesion edge. Again no blocking of FcR were performed. However, FcR bind IgG2b mAbs only weakly, indicating that some monocytes may indeed be present at the border zone in active MS lesions. To confirm that microglia are indeed CD14 negative, we utilized the mAb MY4. This mAb has a broad CD14 reactivity, and stains cells which are not detected by other anti-CD14 mAbs like Leu-M3 (22).

Our results are in agreement with a previous study using anti-lysozyme and MAC 387 to stain formalin fixed MS lesions (18). These authors investigated twenty cases of acute MS, and reported that anti-lysozyme and MAC 387 stained predominantly perivascular cells, with a scattering of positive cells throughout the lesions and at the edge. The majority of foam cells in all lesions remained unstained. Their results indicate that patients with the most recent onset of fatal disease had the greatest number of hematogenous cells in the lesions, indicating a more acute

inflammatory process. Similar results were reported by Esiri and Reading (15) using the markers non specific esterase and RFD7. Both markers stained a proportion of parenchymal cells in the most active lesions, whereas in less active lesions these markers stained perivascular cells only.

Our data from patients with encephalitis are in accordance with a hypothesis suggesting that acute inflammatory activity in the CNS recruits hematogenous phagocytes. The three patients with acute inflammatory encephalitis had monocytes, as identified by MAC 387 and anti-lysozyme, scattered throughout the parenchyma. Monocytes/macrophages were not demonstrated by the same antibodies in the CNS from a patient with chronic encephalitis. Similar results have been reported on frozen sections of CNS from patients with encephalitis using the RFD7 mAb and a mAb reactive with CD14 (UCHM1) (23). Two patients with acute encephalitis had parenchymal cells reactive with UCHM1 and RFD7, whereas these antigens were detectable only on perivascular cells in patients with chronic forms of encephalitis.

Flaris et al. (24) have in a recent paper demonstrated that rat microglia may express de novo a wide variety of molecules shared by monocytes/macrophages in response to distinct CNS activating signals. We do however not believe that the molecules identified on macrophages in our study represent activation markers of human microglia. Support for this comes from 2 lines of evidence: 1) no induction of macrophage-specific antigens could be induced on microglia after activation with IFN-γ. 2) parenchymal cells identified by staining with non specific esterase, CD14, MAC 387, RFD7, and anti-lysozyme were identified mainly in recent encephalitis cases, whereas phagocytically active microglia in most MS lesions were negative for all monocyte/macrophage specific markers.

Various monocyte antigens such as L1 and CD14 are downregulated on mature macrophages (22,25). It can therefore not be excluded that our failure to detect hematogenously derived phagocytes in MS lesions are due to such downregultory mechanisms. However, since also NSE, lysozyme, and the developmentally regulated antigen recognized by RFD7 are all negative on parenchymal cells within most MS lesions, this possibility seems less probable.

Further support for our theory stems from observations made by Lassmann et al. (7) using bone marrow radiation rat chimeras. They demonstrated that hematogenously derived monocytes/macrophages invaded the parenchyma in active lesions of experimental allergic encephalitis (EAE). After recovery from EAE, only a small number of macrophages remained in the CNS. Furthermore, microglia were not replaced by bone marrow derived cells in significant numbers.

Blood vessel-associated myeloid cells have been demonstrated in both rat and human CNS. There is no generally accepted name for these cells, although Graeber and Streit (5) suggested the name perivascular cells to distinguish them from pericytes. It was recently shown that these cells express HLA-DR in histologically normal human brain (26), suggesting that they are of importance for the immunological reactions occuring at the blood-brain interface (6). We have in the present study confirmed previous observations showing that these cells are positive for non specific esterase, CD11c, CD14, CD68, and RFD7 (15,26,27), thus distinguishing them from microglia.

REFERENCES

1. del Rio-Hortega del, P. 1932. Microglia. In Cytology and cellular pathology of the nervous system, vol 2. W. Penfield, ed., Hafner Publishing Company, New York, U.S.A. pp. 481-534.

2. Theele, D.P., and W.J. Streit. 1993. A chronicle of microglial ontogeny. Glia. 7:5-8.

3. Ling, E.A., and W.C. Wong. 1993. The origin and nature of ramified and amoeboid microglia: a historical review and current concepts. Glia. 7:9-18.

4. McGeer, P.L., T. Kawamata, D.G., Walker, H. Akiyama, I. Tooyama, and E.G. McGeer. 1993. Microglia in degenerative neurological disease. Glia. 7:84-92.

5. Graeber, M.B., and W.J. Streit. 1990. Perivascular microglia defined. Trends Neurosci. 13:366-

6. Hickey, W.F. and Kimura, H. (1988) Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vivo. Science, 239, 290-292.

7. Hickey, W.F., K. Vass, and H. Lassmann. 1992. Bone marrow-derived elements in the central nervous system: An immunohistochemical and ultrastructural survey of rat chimeras. J. Neuropathol. Exp. Neurol. 51:246-256.

7. Lassmann, H., M. Schmied, K. Vass, and W.F. Hickey. 1993. Bone marrow derived elements and resident microglia in brain inflammation. Glia. 7:19-24.

8. Williams, K., A. Bar-Or, E. Ulvestad, A. Olivier, J.P. Antel, and V.W. Yong. 1992. Biology of adult human microglia in culture: comparisons with peripheral blood monocytes and astrocytes. J. Neuropathol. Exp. Neurol. 51:538.

9. Hayes, G.M., M.N. Woodroofe, and M.L Cuzner. 1988. Characterization of microglia isolated from adult human and rat brain. J. Neuroimmunol. 19:177.

10. Yong, V.W., and J.P. Antel. 1992. Culture of glial cells from human brain biopsies. In Protocols for Neural Cell culture. A. Richardson, and S. Fedoroff, eds. The Humana Press Inc., St. Louis, MO, U.S.A. 81 - 96.

11. Bøyum, A. 1976. Isolation of lymphocytes, granulocytes and macrophages. Scand. J. Immunol. (Suppl. 5) 5:9.

12. Ulvestad E, Williams K, Vedeler C, Antel J, Nyland H, Mork S, Matre R. Reactive microglia in multiple sclerosis lesions have an increased expression of receptros for the Fc part of IgG. J Neurol Sci (In press).

13. Yam, L.T., C.Y. Li, and W.H. Crosby. 1971. Cytochemical identification of monocytes and granulocytes. Am. J. Clin. Pathol. 55:283.

14. Kaplow, L.S. 1965. Simplified myeloperoxidase stain using benzidine dihydrochloride. Blood. 26:215-219.

15. Esiri, M.M., and M.C. Reading. 1987. Macrophage populations associated with multiple sclerosis plaques. Neurpopathol. Appl. Neurobiol. 13:451-465.

16. Hauser, S.L., Bhan, A.K., F. Gilles, M. Kemp, C. Kerr, and H.L. Weiner. 1986. Immunohistochemical analysis of the cellular infiltrate in multiple sclerosis lesions. Ann. Neurol. 19:578-587.

17. Nyland, H., R. Matre, and S. Mørk. 1980. Fc receptors on microglial lipophages in multiple sclerosis. N. Engl. J. Med. 302:120.

18. Adams, C.W.M., R.N. Poston, and S.J. Buk. 1989. Pathology, histochemistry and immunohistochemistry of lesions in acute multiple sclerosis. J. Neurol. Sci. 92:291-306.

19. Woodroofe, M.N., A.S. Bellamy, M. Feldmann, A.N. Davison, and M.L. Cuzner. 1986. Immunocytochemical characterization of the immune reaction in the central nervous system in multiple sclerosis. Possible role for microglia in lesion growth. J. Neurol. Sci. 74:135-152.

20. Van de Winkel, J.G.J., and C.L. Anderson. 1991. Biology of human immunoglobulin G Fc receptors. J. Leukoc. Biol. 49:511.

21. Hofman, F.M., R.I. von Hanwehr, C.A. Dinarello, S.B. Mizel, D. Hinton, and J.E. Merrill. 1986. Immunoregulatory molecules and IL 2 receptors identified in multiple sclerosis brain. J. Immunol. 136:3239-3245.

22. Ziegler-Heitbrock, H.W.L., and R.J. Ulevitch. 1993. CD14: Cell surface receptor and differentiation marker. Immunol. Today. 14:121.

23. Esiri, M.M., M.C. Reading, M.V. Squier, and J.T. Hughes. 1989. Immunocytochemical characterization of the macrophage and lymphocyte infiltrate in the brain in six cases of human encephalitis of varied aetiology. Neuropathol.Appl. Neurobiol. 15:289-305.

24. Flaris, N.A., T.L. Densmore, M.C. Molleston, and W.F. Hickey. 1993. Characterization of microglia and macrophages in the central nervous system of rats: definition of the differential expression of molecules using standard and novel monoclonal antibodies in normal CNS and in four models of parenchymal reaction. Glia. 7:34-40.

25. Zwaldo G, Bruggen J, Gerhards G, Schlegel R, Sorg C. Two calcium-binding proteins associated with specific stages of myeloid cell differentiation are expressed by subsets of macrophages in inflammatory tissues. Clin exp Immunol 1988:72:510-515.

26. Graeber, M.B., W.J. Streit, D. Büringer, L. Sparks, and G.W. Kreutzberg. 1992. Ultrastructural location of major histocompatibility complex (MHC) class II positive perivascular cells in histologically normal human brain. J. Neuropathol. Exp. Neurol. 51:303-311.

27. Franklin, W.A., D.Y. Mason, K. Pulford, B. Falini, E. Bliss, K.C. Gatter, H. Stein, L.C. Clarke, and J.O'D. McGee. 1986. Immunohistological analysis of human mononuclear phagocytes and dendritic cells by using monoclonal antibodies. Lab. Invest. 54:322-335.

TABLE I

Characteristics of multiple sclerosis and encephalitis patients

Case No.	Autopsy	Age	Sex	Length of illness	Death - post mortem interval (hours)	Diagnosis*
1	O.570/86	41	F	7 years	8	MS
2	O.756/91	56	F	26 years	24	MS
3	R.106/92	42	F	10 years	41	MS
4	O.614/92	43	M	7 years	45	MS
5	O.737/79	46	F	2 years	27	MS
6	O.205/77	33	M	7 days	30	E
7	O.260/92	18	M	10 days	19	E
8	O.368/82	51	M	8 days	30	E
9	R.175/92	5	M	4 months	25	CEBVE

*MS, Multiple Sclerosis; E, Encephalitis;

CEBV, Chronic Epstein Barr virus encephalitis.

Table II

Antibodies used in present study

Antigen	MoAb/Ab	Isotype	Reactivity pattern	Manufacturer†	Dilution
CD11c	LeuM5	IgG2b	Monocytes/macrophages	B&D	1 μg/ml
CD14	TÜK4	IgG2a	Monocytes/macrophages	Dako	5 μg/ml
CD14	LeuM3	IgG2b	Monocytes/macrophages	B&D	5 μg/ml
CD14	AML-2-23	IgG2b	Monocytes/macrophages	Medarex	5 μg/ml
CD14	MY4	IgG2b	Monocytes/macrophages	Coulter	5 μg/ml
CD21	1F8	IgG1	B cells	Dako	1:10
CD21	Anti-CR2	IgG2a	B cells	B&D	25 μg/ml
CD45	PD7/26	IgG1	Leukocytes	Dako	1:10*
CD64 (FcRI)	32.2	IgG1	Monocytes/macrophages	Medarex	5 μg/ml
CD68	KP1	IgG1	Monocytes/macrophages	Dako	1:50*
L1	MAC 387	IgG1	Monocytes/macrophages	Dako	1:50*
Lysozyme	Lysozyme	Poly	Monocytes/macrophages	Dako	1:1000*
Undesignated	RFD7	IgG1	Tissue macrophages	L. Poulter	1:5

†B/D: Becton Dickinson, Mountain View, Cal., USA. Dako: Dakopatts, Glostrup, Denmark. Medarex Inc., West-Lebanon, NH, USA. Coulter Immunology, Hialeah, FL, U.S.A.
L. Poulter: Royal Free Hospital, London, UK.

*Suitable for use on formalin-fixed tissue.

Table III

Markers used to differentiate between microglia, monocytes, macrophages, and perivascular cells.

	Microglia (time in culture)		Monocytes (time in culture)		Perivascular cells
Markers	in situ	8 days	60 min	8 days	in situ
NSE	-	-	+++	+++	+
MPE	-	-	+++	-	-
L1	-	-	+++	+++	-
Lysozyme	-	-	+++	+++	-
RFD7	-	-	-	++	+
CD11c	+	+++	+++	+++	+
CD14	-	-	+++	+++	+
CD68	+	+++	+++	+++	+

- = Negative; + = positive cells identified <u>in situ</u>; ++ = 30-40% positive cells in culture;

+++ = more than 70% of cells in culture positive. NSE = non specific esterase. MPE = myeloperoxidase.

FIGURE LEGENDS

Figure 1. a,b: RFD7 on perivascular cell in normal CNS (x670). c,d: Perivascular cell in normal CNS identified by a mAb to CD14 (Leu-M3) (x420). e: CD58 staining of microglia in normal CNS (x330). f: Non-specific esterase staining of perivascular and occassional parenchymal cells (arrow) in MS lesion (x330). Counterstaining with hematoxylin.

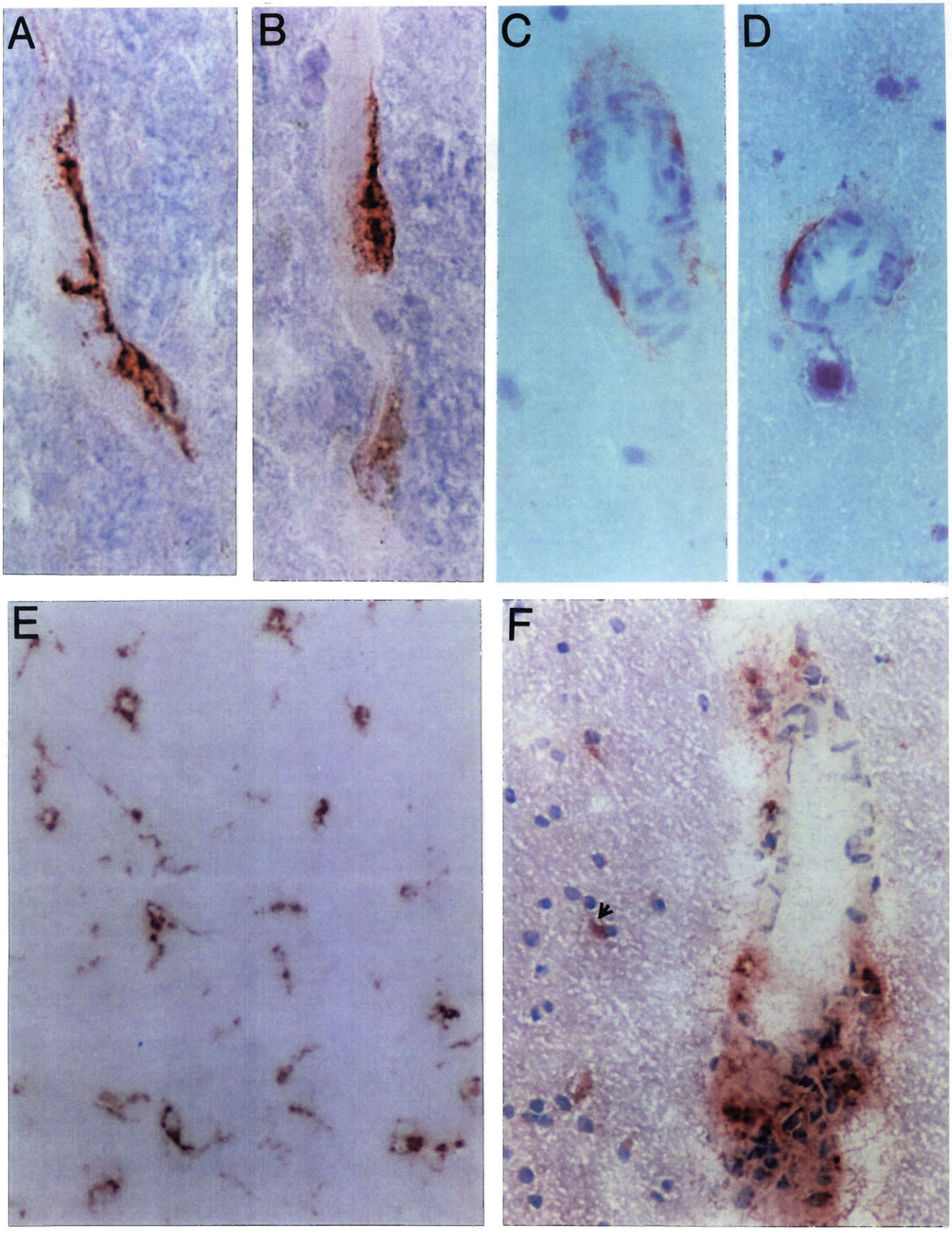

A
B
C
D
E
F

Figure 2. a,b: RFD7 reactive perivascular cells in active MS lesion (A; x170, B: x420). c: Leu-M3 staining on microglia in the normal CNS. d: Reactivity with IgG2a isotype control mAb in unblocked MS lesions (x420). e: CD11c staining on microglia in MS lesion (l) and in surrounding white matter (w) (x330). Counterstaining with mematoxylin.

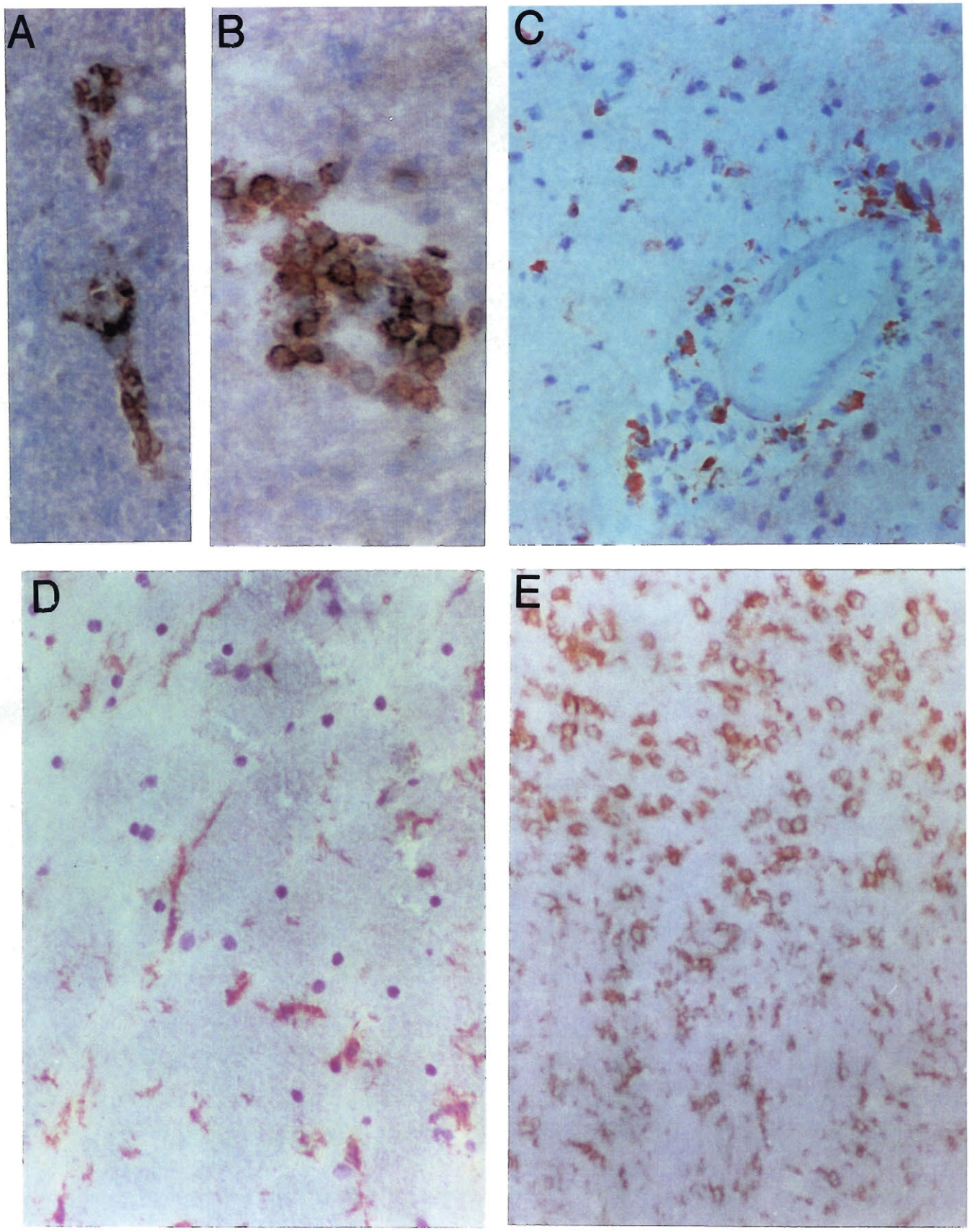
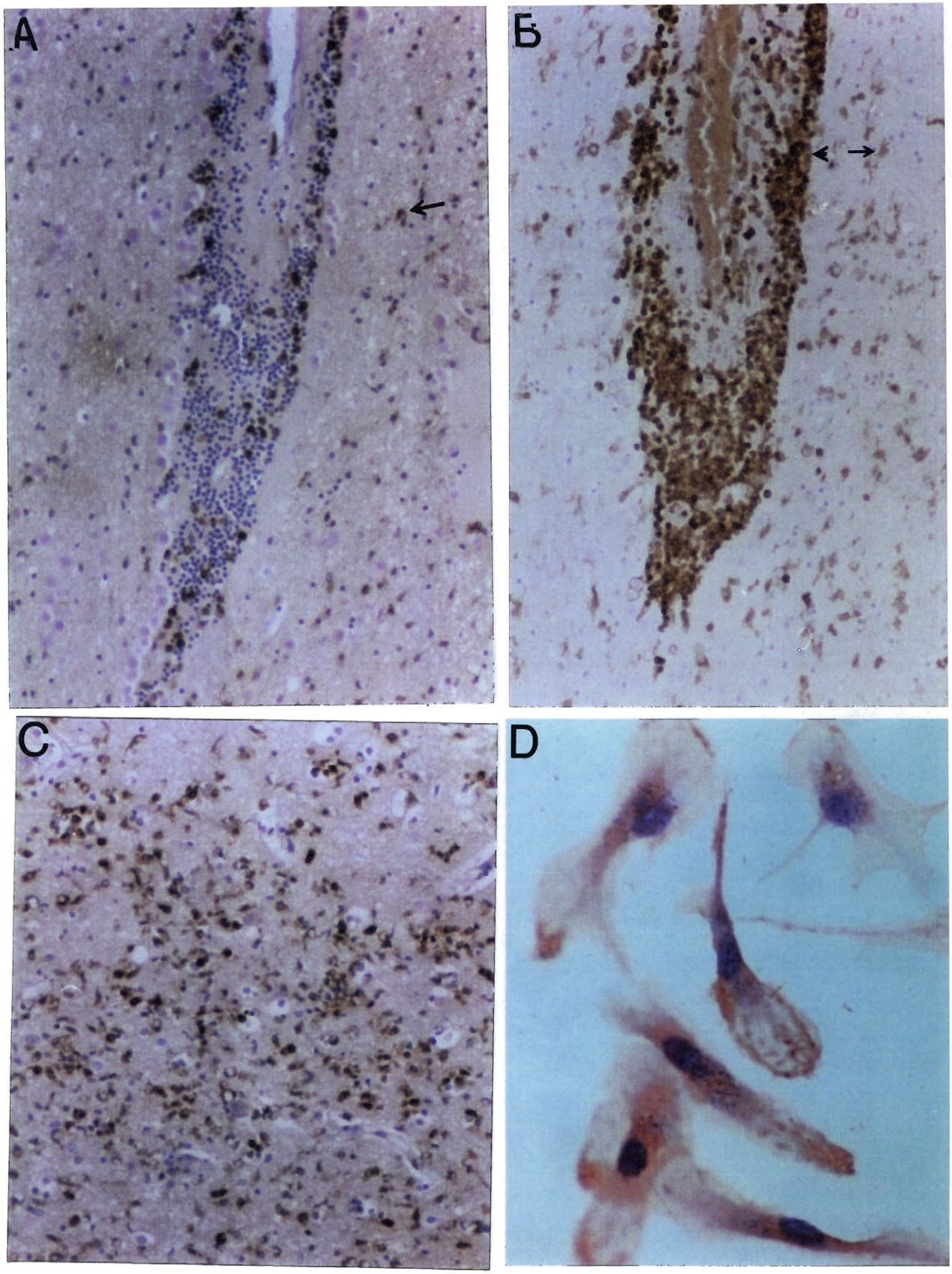

A
B
C
D
E

Figure 3. a: MAC387 reactivity on perivascular monocytes/macrophages in encephalitis brain. Note occassional positive cells in parenchyma (arrow) (x170). b: CD45 reactivity on perivascular and parenchymal leukocytes (large arrow) and parenchymal microglia (small arrow) in encephalitis brain (x170). c: Lysozyme staining of cells invading the parenchyma in encephalitis brain (x170). d: CD45 positive cultured microglia (x420). Counterstaining with hematoxylin.


A
Б
C
D

Part II

Immune accessory functions

Chapter 4

INDUCTION OF PRIMARY T CELL RESPONSES BY HUMAN GLIAL CELLS

Kenneth Williams, Elling Ulvestad, Manon Blain, and Jack P. Antel

Preface

Following the observation that microglia expressed a high level of MHC class II antigens in vitro over that of autologous astrocytes and that microglia could present recall antigens to freshly isolated T cells, it was of interest to determine if microglia could function as antigen presenting cells initiating a primary immune response in a mixed lymphocyte reaction system (MLR). Major points demonstrated in this section include:

1. In addition to the expression of MHC class II antigens on microglia in vitro, MHC class II antigens could also be demonstrated on snap frozen tissue from the same specimens used to established microglial cultures. No MHC class II positive astrocytes were demonstrated in the same surgical specimens.

2. Mixed glial cell cultures consisting of microglia, astrocytes, and oligodendrocytes, were able to present antigen to freshly isolated $CD4^+$ T cells in a primary mixed lymphocyte reaction system.

3. Microglia and astrocytes both were immunoreactive for the immune accessory molecules HLA-DR and LFA-3, while only microglia were positive for LFA-1 and B7/BB-1, particularly following IFN gamma stimulation.

4. Using enriched microglial and astrocyte cultures, it was found that microglia alone were capable of presenting antigen in the primary mixed lymphocyte reaction system. Astrocytes even following the induction of HLA-DR, could not function as an antigen presenting cell in this assay.

ABSTRACT

Glial cells of the central nervous system (CNS) are postulated to function as immune accessory cells which may regulate immune reactivity occurring within the CNS, activating or alternatively inhibiting T cell responses. We have utilized surgically resected cerebral tissue derived from young adult humans to prepare dissociated cultures of glial cells (mixed astrocyte-microglia-oligodendrocyte cultures) and demonstrate that such cells are capable of acting as stimulators of primary T cell responses, using proliferation of T cells to allogeneic determinants on the glial cells as the test system. Studies of resected adult cerebral tissue indicated major histocompatibility complex (MHC) class II antigen expression on microglia in situ. Using a mixed lymphocyte reaction (MLR), we observed that enriched microglial cultures alone were capable of stimulating primary responses of freshly isolated T cells or the $CD4^+$ T cell subset, a response which could be inhibited with an anti-MHC class II blocking antibody. In agreement with previous studies using rodent-derived astrocytes, we found that human astrocytes (fetal), could not initiate a primary T cell response even after up-regulation of MHC class II antigen expression with interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα). Our results indicate that a primary T cell response, as well as a secondary response to a recall antigen, can occur within the CNS; our data implicate microglia as the central cell involved in the former.

INTRODUCTION

The role of glial cells in regulating immune reactivity within the CNS remains under active study. These cells include astrocytes, a cell type defined by the expression of glial fibrillary acidic protein (GFAP) and microglia, a cell type expressing many but not all phenotypic markers of cells from the haematopoietic lineage (1-3). Under non-pathologic conditions, human astrocytes appear not to express major histocompatibility complex (MHC) molecules ***in situ***, whereas such expression on human microglia is reported (4-8). Although MHC class II expression on astrocytes has been described ***in situ*** under pathologic conditions (9,10), microglia are most probably the major cell expressing class II molecules under these conditions

(5,11). Under basal tissue culture conditions a proportion of human astrocytes express MHC class II molecules (12), whereas expression of MHC class II is found on the majority of adult human microglia (3). Treatment of astrocytes and microglia with T cell-produced cytokines, including interferon gamma (IFNγ), and tumor necrosis factor alpha (TNFα), induces or up-regulates MHC class II expression (3, 12-13).

The capacity of glial cells to present antigen to T cells, with subsequent T cell activation and proliferation, has received previous attention. T cell activation requires both antigen-MHC interaction with the T-cell receptor (TcR) and secondary signals delivered by the antigen-presenting cell (APC). Evidence that glial cells can present antigen is based upon the observations that: (a) antigen presentation by rodent astrocytes and microglia pre-stimulated with IFNγ results in continued proliferation of previously sensitized T cell lines (15,16) and (b) unstimulated human microglia can present recall antigens, resulting in the proliferation of freshly-isolated autologous human T cells. This latter response is further upregulated when microglia are pre-stimulated with IFNγ (3). Antigen presentation by rodent astrocytes to naive T cells does not result in T-cell activation (17).

In the present study, we have examined the capacity of human glial cells to present antigen to unprimed T cells ***in vitro***. For these studies we have used freshly-derived unfractionated human T cells and purified $CD4^+$ cells, the T cell subset implicated as the primary antigen-specific autoreactive T cell and the subset known to respond to allogeneic MHC class II (18). In initial experiments we observed a proliferative response of whole mononuclear cells (MNC) to dissociated mixed glial cell cultures comprised of astrocytes, microglia, and oligodendrocytes. To further define the relative capacity of individual glial cell populations to present antigen, we used enriched cultures of adult human microglia and fetal human astrocytes. Highly enriched microglial preparations can be prepared from the initial mixed glial cultures, based on their differential adhesion properties (3,19). We were unable to prepare cultures of human adult astrocytes of sufficient purity or cell number for these studies and therefore utilized fetal human astrocyte cultures. Our results indicate that

microglia represent an endogenous CNS cell capable of priming T cell responses. Astrocytes, even when induced to up-regulate expression of MHC class II antigens, are not able to initiate T cell activation.

METHODS AND METHODS

Source of glial cells - Adult human glial cell cultures were prepared from cerebral cortical tissue resected from young adults (N=7) (ages 18-35) undergoing surgical therapy for treatment of intractable epilepsy. Cases in which tumor or vascular malformation was found were excluded. Neuropathological examination of the tissue showed varying extent of gliosis, ranging from normal to severe. The bulk of the tissue used in our studies was derived from regions requiring resection to reach the precise epileptic focus, and was distant from the main electrically-active site. Fetal human glial (astrocyte) cultures were prepared from 8-12-week old specimens (N=12) following Medical Research Council of Canada approved guidelines.

Source of lymphocytes: - Peripheral blood mononuclear cells (PBMC) were derived from individuals from whom surgical cerebral tissues were available (autologous) or from young adult normal volunteer donors (allogeneic). Splenic tissue from fetuses was used to obtain fetal lymphoid preparations.

Glial cell preparations

Adult - Our method for preparing human mixed glial cell cultures has been described previously (12,19). Briefly, tissues were treated with 0.25% trypsin and DNAse (50 μg/ml), followed by Percoll (Pharmacia LKB Biotechnology, Uppsala, Sweden) gradient centrifugation at 15,000 rpm for 30 minutes (min.). Cells were then suspended in culture medium, consisting of Eagle's minimal essential medium (MEM) supplemented with 5% fetal bovine serum (FBS), 0.1% glucose, and 20 μg/ml gentamicin (Gibco, Burlignton, Ontario). Two types of cultures were prepared from this initial isolate. A) Mixed glial cultures- for these, the initial cell isolates were

either seeded directly into 96 well microtiter plates or onto 9 mm Aclar fluorocarbon coated coverslips (Dr. S. Kim, Vancouver, British Columbia) previously coated with 10 μg/ml poly-l-lysine (Sigma, St. Louis, USA). These mixed glial cultures contained microglial cells in addition to oligodendrocytes and astrocytes. B) Microglia enriched cultures: to enrich for microglia, the initial cell isolates were plated into uncoated 25 cm^2 Nunc flasks (Gibco). Oligodendrocytes remained floating and were removed the following day. The adherent properties of adult human microglia cells contrast to thoses of the neonate rodent preparations or human fetal preparations where neonate or fetal cells are less adherent than astrocytes and thus can be floated off of the initial cultures. To isolate adult human microlgia, the adherent cells consisting of microglia and astrocytes were allowed to develop morphologically for seven days, and the astrocytes were then floated off by rotary shaking for 5 hours at 150 rpm. The human microglial cells were then detached with 0.25% trypsin and DNAse (50 μg/ml) at 37°C for 15 min., and then seeded onto coverslips or into 96 well microtiter plates.

Fetal - These cultures were prepared by dissociation of fetal cerebral tissue (8-12 week) with trypsin (0.05%), passing the tissue through a 125 μm nylon mesh, washing 2X in phosphate buffered saline (PBS), and plating directly onto poly-L-lysine-coated plastic culture dishes (20). Cells were trypsinized with 0.05% trypsin and 50 μg DNAse for 15 min. at 37°C and split when confluent ($\approx$ every 2 weeks). Experiments were conducted after the second or third cell passage when fetal neurons were no longer apparent.

Immunocytochemical studies

In-vitro- The purities of microglia and astrocyte cultures were determined by immunostaining with mouse anti-CD11c (Leu-M5) (1:10) (Becton Dickinson, San Jose, CA) or rabbit anti-GFAP Ab (1:100) (Dako, Westchester, PA), respectively followed by rhodamine conjugated goat anti-mouse antibodies (1:150) (Cappel, Lexington, MA) or fluoroscein conjugated goat anti-rabbit antibodies (1:150) (Cappel). The anti-GFAP

staining required fixation of cells in acid alcohol (5% glacial acetic acid:95% absolute ethanol, v/v). In all staining protocols, sister culture coverslips were used as controls with HHG (2% horse serum, 1mM Hepes, and 10% goat serum in Hanks balanced salt solution) substituting for the primary antiserum. The HHG was the diluting medium of all antibodies. Except for fixation at -20°C all procedures were done at room temperature. Proportions of immunostained cells were assessed either by counting using a fluorescent microscope or by FACScan (Becton-Dickinson).

Immune-accessory molecule expression was assessed on enriched microglial and fetal astrocyte cultures under basal and activation conditions. For these studies cells on coverslips were washed 3X with PBS followed by incubation with primary antibody against CD11a (LFA-1) (1:100) (AMAC Inc., Westbrook, ME), CD58 (LFA-3) (1:100) (AMAC), CD54 (ICAM-I) (1:100) (Bio Design International, Kennebunkport, ME) and B7/BB-1 (1:100) (Becton-Dickinson) for 45 min. Coverslips were then washed 3X with PBS and incubated with rhodamine conjugated goat anti-mouse Ig for 45 min. Coverslips were again washed and the cells were fixed and stained for GFAP immunoreactivity as described above. Staining for HLA-DR (mAb D1-12, 1:1000, a gift from Dr. R. Sekaly, CRIM, Montreal, PQ) was assessed using a HLA-DR, GFAP double staining procedure as previously described (Williams et al., 1992).

In situ - 4-6μm sections were prepared from cerebral tissue specimens which were derived from surgical resections which had been quick-frozen in liquid nitrogen, blocked in Tissue Tek (Miles, Elkhart, IN), and stored at -70 degrees C. Serial tissue sections were fixed in cold acetone for 5 minutes and washed in PBS. Endogenous peroxidase was inactivated by pre-treatment with 0.3% H_2O_2 for 10 minutes. Sections were thereafter washed in PBS and incubated overnight at 4°C in a humid chamber with Leu M5 (1:10) or anti-HLA class II (SG465) mAb (1:50) (a gift from Dr. R. Sekaly, Montreal, Quebec). Biotinylated rabbit anti-mouse immunoglobulins (Dakopatts, Glostrup, Denmark) diluted 1:300 were applied for 30 minutes. Avidin-biotin peroxidase complex (ABComplex/HRP, Dakopatts) was prepared as

recommended by the manufacturer and allowed to react with sections for 30 minutes. Sections were thereafter washed in PBS and treated with a 3-amino-9-ethyl-carbazole-containing buffer for the development of a colored reaction product.

Preparation of lymphocyte populations

Mononuclear cells (MNC) were isolated from heparinized peripheral blood (PB) samples obtained from adult donors by Ficoll-Hypaque (Pharmacia, LKB) density centrifugation. Total T cells (E^+ cells) were isolated by rosetting with S-(2-Aminoethyl) isothiouronium bromide hydrobromide (AET)-treated sheep red blood cells (RBC) (21). Non-rosetting cells (E^- cells) consisting of monocytes and B-cells, were used as APC's in some experiments described below. $CD4^+$ T cell populations were prepared by incubating total T cells with anti-CD8 monoclonal antibodies (mAbs) (prepared from OKT8 hybridoma obtained from ATCC, Rockville, MD), followed by addition of rabbit complement (Cedarlane Laboratories, Hornby, Ontario) (21,22). Purity of total T cell and $CD4^+$ T cell preparations was assessed by immunostaining with fluorescein-labelled anti-CD8 mAb (Leu2) (Becton-Dickinson) and phycoerythrein conjugated anti-CD4 mAb (Leu3) (Beckton-Dickinson) and FACScan analysis (Beckton-Dickinson). MNC's were prepared from fetal spleen tissue by dissociating the tissue, passing it through a nylon mesh, lysing RBCs with Guy's hypotonic saline followed by Ficoll-Hypaque gradient centrifugation. Lymphocytes were cultured in medium comprised of RPMI supplemented with 5% human AB (Pel-Freze, Brown Deer, WI) or autologous serum, 2.5μg/ml penicillin, and 2.5μg/ml streptomycin (Gibco).

Functional Lymphocyte Assays:

Priming responses- $1x10^5$ allogeneic or autologous lymphocytes (either MNC's, total T cells, or $CD4^+$ T cells) were placed into individual wells of 96 well flat-bottom microtiter plates (Nunc) into which radiated glial cells had been seeded at a concentration of $2x10^4$ cells per well. Glial cells were irradiated in the 96 well plates

with 2500 rads (AECL Gammacell 1000 irradiator) prior to the addition of lymphoid cells. The fetal astrocytes were utilized under basal culture conditions and after stimulation with recombinant human IFNγ (100 U/ml) (Boehringer Mannheim, Germany) and TNF-∝ (100 U/ml) (Genzyme, Boston, MA) 48 hours prior to lymphocyte coculture. Wells were rinsed 2X with culture medium prior to the addition of T cells.

For mixed lymphocyte reactions (MLR) in which fetal spleen cells were used as stimulators, these cells were irradiated (2500 rads) and used at $1x10^5$ cells per microwell. Experiments were also performed in which freshly-isolated $CD4^+$ T cells were cocultured with allogeneic E^- cells at a 1:1 ratio in the presence of $1\text{-}2x10^4$ IFNγ/TNF∝-treated irradiated astrocytes. In some experiments, a blocking antibody against HLA-DR (L243) (1:300) (a gift from Dr. R. Sekaly, Montreal, Quebec) was preincubated with the microglia for 1 hour prior to the addition of T cells.

Recall antigen responses - In initial studies, T cells were cocultured with $10^3\text{-}10^4$ autologous microglia plus Candida albicans proteins diluted 1:10,000 from a stock solution (4030 UI/ml stock, Rhone-Poulenc Pharma, Montreal, PQ). Subsequent experiments were performed in which $CD4^+$ T cells, initially cultured with $1\text{-}2x10^4$ stimulated allogeneic fetal astrocytes were recovered, washed, and cultured with fresh autologous irradiated MNCs as a source of APC's plus tetanus toxoid antigen (1:100 final volume dilution of 50 protein units per ml stock, a gift from Dr. K. Osterland, Royal Victoria Hospital, Montreal, PQ).

Mixed lymphocyte reaction (MLR) functional assays were carried out for 5 days and recall antigen responses were assessed after 7 days. Proliferative responses were determined by pulsing cultures for 5 hours with ^{3}H-thymidine (1 μCu per well) (ICN Flow Laboratories, Mississagua, Ontario), harvesting the wells, and counting radioactivity using a beta liquid scintillation counter (LKB, Fisher, Montreal). Results were calculated as mean counts per minute (CPM) of triplicate wells.

RESULTS

As shown in Table 1., dissociated mixed glial cell cultures (50-70% $GFAP^+$ cells) were able to induce significant proliferation of unfractionated allogeneic MNC's. In the mixed glial preparations, the majority of microglia and some astrocytes (10-20%), but none of the oligodendrocytes, were positive for HLA-DR expression. Figure 1 demonstrates ***in situ*** expression of MHC class II molecules on microglia. As summarized in Table 2., microglia in vitro expressed LFA-1, LFA-3, and B7/BB-1 molecules, particularly under activating conditions; fetal astrocytes were not LFA-1 or B7/BB-1 immunoreactive.

To assess which of the individual glial cell populations could induce activation of allogeneic lymphocytes, enriched microglial cultures were prepared from adult donors and astrocytes from fetal CNS tissue (Fig. 2, Fig. 3). As shown in Figure 4, microglia could induce significant proliferation of $CD4^+$ T cells prepared from allogeneic donors compared to the response of responder or stimulator cells cultured alone or to autologous $CD4^+$ T cell/microglia cocultures. ^{3}H-thymidine incorporation by $CD4^+$ T cells cocultured with autologous microglia was greater than that by $CD4^+$ T cells cultured alone. Prior incubation of microglia with anti-HLA-DR antibody (mAb L243) significantly reduced the microglia-induced proliferation of allogeneic T cells (Fig. 5). Microglia were also able to present recall antigens to autologous T cells (Table 3).

In contrast to the microglia, fetal astrocytes did not induce proliferation of allogeneic T cells, even if the astrocytes were pre-treated with IFNγ (100 U/ml) and TNFα (100 U/ml) (Fig. 4). Pre-treatment of the astrocytes with IFNγ and TNFα did increase MHC class II expression on the cells (Fig. 3). Irradiated spleen-derived MNCs from the same fetal tissue that served as the source of astrocytes did induce significant proliferation of allogeneic T cells (Fig. 4).

To investigate whether astrocytes exerted an inhibitory effect on the T cells, MLR's were performed in which $CD4^+$ T cells and allogeneic E- were cocultured on a layer of stimulated fetal astrocytes. ^{3}H-thymidine uptake by 10^5 $CD4^+$ T cells

cocultured with 10^5 allogeneic E^- cells (1:1 ratio) and 1-2x10^4 irradiated astrocytes was comparable to that of $CD4^+$ T cells co-cultured only with allogeneic E^- cells (73639 $\pm$ 17845 cpm vs. 74114 $\pm$ 17126 SEM) (N=7). In additional experiments, we found that pre-culturing $CD4^+$ T cells with allogeneic stimulated astrocytes for 48 hours did not significantly inhibit the response of the T cells to subsequent stimulation by tetanus toxoid presented by irradiated autologous MNC's (data not shown).

DISCUSSION

The capacity of glial cells to promote or alternatively inhibit immune reactivity within the CNS has been considered a potentially important element in the development and course of autoimmune responses within the CNS. Whether IL-2 production and T cell proliferation follows presentation of antigen by an APC is dependent both on the pre-existent activation state of the T cell and the properties of the APC (23,24). Antigen/T cell receptor interaction without optimal co-signals being delivered to the T cell can induce a state of non-responsiveness of the T cell (anergy) (24-26) or programmed cell death (apoptosis) (27,28). In the current study, we have assessed the ability of human glial cells to activate or prime T cells using the MLR system. Using mixed glial cell cultures, we observed proliferation of both unfractionated allogeneic MNC's and enriched $CD4^+$ T cell preparations, even without cytokine pre-treatment of the glial cultures. At the stimulator: responder ratio used (1:5), only a low level of autologous T cell responses were observed. Both microglia and a variable proportion of the astrocytes in these cultures express MHC class II molecules. Graeber et al. (8) and Lampson and Hickey (4) report the expression of MHC class II molecules on microglia in the parenchyma ***in situ***, using autopsy material of "normal" brain. Similarly, we observed MHC class II molecule expression on microglia, but not on astrocytes, in the surgically-derived material from which our microglial cultures were established.

To define which of the glial elements accounts for the priming effect, we utilized enriched (>95%) dissociated cultures of either adult human microglia cells or

fetal human-derived astrocytes. In the current study we observed that microglia can present antigen to unprimed $CD4^+$ T cells, with resultant T cell activation and proliferation. These observations extend previous reports (3,16,29) that microglia can present antigen to antigen-primed T-cell lines and recall antigens to freshly-isolated peripheral blood T cells. Although bone marrow-derived dendritic cells are considered the main (most potent) stimulators of the MLR (30), macrophages, endothelial cells, and B cells also are reported to be competent APC's in this system. However, macrophages are reported to stimulate $CD4^+$ T cells only following stimulation of the macrophages with IFN and LPS (31,32) , raising the issue as to whether microglia are more efficient as APCs than other resident macrophages. Microglia, similar to resident macrophages in other tissues, express many but not all monocyte cell-associated markers (3); they also do not express all markers associated with dendritic cells.

Our data using human fetal astrocytes as APC's are similar to previous analyses of immune accessory properties of human and rodent astrocytes. We observe a variable level of MHC class II molecule expression by these cells in vitro. Class II expression is further augmented when these cells are treated with IFNγ and TNF-α. In agreement with the results of Sedgwick et al. (17), we find that the astrocytes, even after up-regulation of class II molecules, do not prime $CD4^+$ T cell responses. Sedgwick et al. (17), in accord with previous reports by Fontana et al. (33), did observe that rodent astrocytes could support proliferation of activated $CD4^+$ T cell lines; Matsumoto et al. (16), however, reported that rodent astrocytes did not support such a response and suggested that astrocytes may actively inhibit responsiveness. Similarly, Meinl et al. (34) report that fetal human astrocytes do not support proliferation of myelin basic protein (MBP)-primed T cell lines and also observed an inhibitory effect on proliferating T cells. We found that $CD4^+$ T cells cocultured or pre-cultured for 48 hours with allogeneic fetal astrocytes could still be activated either by allo-antigen or by recall antigen presented by autologous APC's, respectively. Previous reports indicate that T cell clones stimulated with non-bone marrow-derived

class II-expressing APCs (35) or metabolically inactive APC's (23) do not produce IL-2, but instead become anergic. Whether astrocytes which express class II antigen but may lack necessary secondary signals to drive T cells to proliferate but instead induce anergy in autologous T cells, remains to be established.

The precise factors accounting for the observed functional stimulatory differences between microglia and astrocytes remain to be resolved; donor age differences alone do not account for the failure of fetal astrocytes to prime since fetal spleen cells from the same donor source were potent stimulators in the same assay. Expression of class II molecules on the APC is the minimal requirement for antigen presentation to class II-restricted $CD4^+$ T cells. Class II antigen expression under both basal and cytokine stimulation conditions is greater on microglia than on autologous adult human astrocytes (3). The microglia-stimulated MLR is inhibited by the anti-HLA-DR antibody L243 (36). Additional APC properties which favor T cell activation include interactions between accessory adhesion molecules and production of cytokines by the APC. In agreement with previous reports we found that fetal human astrocytes express LFA-3 and ICAM, but not LFA-1 (37). Microglia expressed all three of these molecules. We further observed that microglia, particularly following activation, expressed B7/BB1, an antigen recently demonstrated to be expressed on B cells (38) and activated macrophages (39), and to be involved in the stimulation of T cells in the MLR (38)

Cytokine co-stimulatory signals which can contribute to the induction of a MLR include IL-1, IL-6 and TNF; each of these cytokines has been reported to be produced by astrocytes and microglia (40,41). However, the relative amount of synthesis by microglia and astrocytes of cytokines like IL-1, and the levels of surface-bound IL-1 expressed by these two cell populations is not resolved and remains controversial (42,43). Functionally it has been shown that ligation of LFA-3, CD44, and CD45 surface molecules on monocytes can stimulate these cells to release IL-1 and TNF, contributing to T cell activation (44).

Our results were obtained using human glial cell cultures prepared from

surgically derived CNS tissue. Previous studies have indicated both species and age-related variables in the biologic properties of both microglia and astrocytes (20). The capacity of human adult glial cells, particularly microglia, to both induce and sustain immune responses within the CNS could contribute to the often relapsing, characteristically progressive, course of the putative autoimmune disease multiple sclerosis. Such glial cell properties may be amenable to therapeutic intervention by immuno-modulatory agents, some of which are the subject of current or proposed clinical trials.

References

1. Perry VH, Gordon S (1988): Macrophages and microglia in the nervous system. TINS 11:273-277.

2. Hayes GM, Woodroofe MN, Cuzner ML (1988): Characterisation of microglia isolated from adult human and rat brain. J Neuroimmunol 19:177-189.

3. Williams K, Bar-Or A, Ulvestad E, Olivier A, Antel JP, Yong VW (1992): Biology of adult human microglia in culture: comparisons with peripheral blood monocytes and astrocytes. J Neuropath Exp Neurol 51:538-549.

4. Lampson LA, Hickey WF (1986): Monoclonal antibody analysis of MHC expression in human brain biopsies:tissue ranging from "histologically normal" to that showing different levels of glial tumor involvement. J Immunol 136: 4054-4062.

5. Hayes GM, Woodroofe MN, Cuzner ML (1987): Microglia are the major cell type expressing MHC class II in human white matter. J Neurol Sci 80:25-37.

6. Mattiace LA, Davies P, Dickson DW (1990): Detection of HLA-DR on microglia in the human brain is a function of both clinical and technical factors. Am J Pathol 136:1101-1114

7. Dickson D W, Mattiace L A, Kure K, Hutchins K, Lyman WD, Brosnan CF (1991): Biology of disease: microglia in human disease, with an emphasis on acquired immune deficiency syndrome. Lab Invest 64: 135-156.

8. Graeber MB, Streit WJ, Buringer D, Sparks DL, Kreutzberg GW (1992): Ultrastructural location of major histocompatibility complex class II positive perivascular cells in histologically normal human brain. J Neuropath Exp Neurol 51:303-311.

9. Traugott U, Scheinberg LC, Raine CS (1985): On the presence of Ia-positive endothelial cells and astrocytes in multiple sclerosis lesions and its relevance to antigen presentation. J Neuroimmunol 8:1-14.

10. Lee SC, Raine CS (1989): Multiple sclerosis: oligodendrocytes in active lesions do not express class II major histocompatibility complex molecules. J Neuroimmunol 25:261-266.

11. Matsumoto Y, Hara N, Tanaka R, Fujiwara M (1986): Immunohistochemical analysis of the rat central nervous system during experimental allergic encephalomyelitis, with a special reference to Ia-positive cells with dendritic morphology. J Immunol 136:3668-72.

12. Yong VW, Yong FP, Ruijs TCG, Antel JP, Kim SU (1991): Expression and modulation of HLA-DR on cultured human adult astrocytes. J Neuropathol Exp Neurol 50:16-28.

13. Wong GHW, Bartlett PF, Clark-Lewis I, Battye F, Schader JW (1984): Inducible expression of H-2 and Ia antigens on brain cells. *Nature* 310:688-696.

14. Dhib-Jalbut S, Kufta CV, Flerlage M, Shimojo N, McFarland HF (1990): Adult human glial cells can present target antigens to HLA-restricted cytotoxic T-cells. J Neuroimmunol 29:203-211.

15. Fontana A, Fierz W, Wekerle H (1984): Astrocytes present myelin basic protein to encephalitogenic T-cell lines. Nature 307:273-276.

16. Matsumoto Y, Ohmori K, Fujiwara M (1992): Immune regulation by brain cells in the central nervous system: microglia but not astrocytes present myelin basic protein to encephalitogenic T cells under in vivo-mimicking conditions. Immunology 76: 209-216.

17. Sedgwick JD, Mößner R, Schwender S, ter Melen V (1991): Major histocompatibility complex-expressing non-hematopoietic astroglial cells prime only $CD8^+$ T lymphocytes: Astroglial cells as perpetuators but not initiators of $CD4^+$ T cell responses in the central nervous system. J Exp Med 173:1235-1246.

18. Sprent J, Schaefer M (1989): Antigen-presenting cells for unprimed T cells. Immunol Today 10:17-23.

19. Yong VW, Antel JP (1992a): Culture of glial cells from human brain biopsies. In Richardson A, Fedoroff S (eds.): "Protocols for Neural Cell Culture." St. Louis:Humana Press, 81-96.

20. Yong VW, Tejada-Berges T, Goodyer CG, Antel JP, Yong FP (1992b): Differential proliferative response of human and mouse astrocytes to gamma-interferon. Glia 6:269-280.

21. Pellegrino MA, Ferrone S, Theofilopoulos AN (1976): Isolation of human T and B lymphocytes by rosette formation with 2-aminoethylisothioquronium bromide (AET)-treated sheep red blood cells. J Immunol Methods 11:273-279.

22. Ruijs TCG, Louste K, Brown EA, Antel JP (1993): Lysis of human glial cells by major histocompatibility complex-unrestricted $CD4^+$ cytotoxic lymphocytes. J Neuroimmunol 42:105-111.

23. Jenkins MK, Schwartz RH (1987): Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. J Exp Med 165: 302-319.

24. Schwartz RH (1990): A cell culture model for T lymphocyte clonal anergy. Science 248: 1349-1356.

25. DeSilvia DR, Urdahl KB, Jenkins MK (1991): Clonal anergy is induced in vitro by T cell receptor occupancy in the absence of proliferation. J Immunol 147: 3261-3267.

26. Jenkins MK, PArdoll DM< Mizuguchi J, Chused TM, Schwartz RH (1987): Molecular events in the induction of a nonresponsive state in interleukin-2 producing helper T lymphocyte clones. Proc Ntal Acad Sci (USA) 84: 5409-5413.

27. Newell MK, Haughn LJ, Maroun CR, Julius MH (1990): Death of mature T cells by seperate ligation of CD4 and the T cell receptor for antigen. Nature:286-289.

28. Pender MP, McCombe PA, Yoong G, Nguyen KM (1992): Apoptosis of α, β T lymphocytes in the nervous system in experimental autoimmune encephalomyelitis: Its possible implications for recovery and acquired tolerance. J Autoimmunity 5:401-410.

29. Frei K, Siepl C, Groscurth P, Bodmer S, Schwerdel C, Fontana A (1987): Antigen presentation and tumor cytotoxicity by interferon-γ-treated microglial cells. Eur J Immunol 17:1271-1278.

30. Steinman RM (1991): The dendritic cell system and its role in immunogenicity. Annu Rev Immunol 9:271-296.

31. Inaba K, Steinman RM (1984:) Resting and sensitized T lymphocytes exhibit distinct stimulatory (antigen-presenting cell) requirements for growth and lymphokine release. J Exp Med 160:1717-1735.

32. Metlay JP, Pure E, Steinman RM (1989): Control of the immune response at the level of the antigen-presenting cells: a comparison of the function of dendritic cells and B lymphocytes. Adv Immunol 47:45-56.

33. Fontana A., Fierz W., Wekerle H (1984):Astrocytes present myelin basic protein to encephalitogenic T-cell lines. Nature 307:273-276.

34. Meinl W, Weber F, Aloisi A, Hohlfeld B, Werkerle H (1991): Interactions of human astrocytes and autoreactive T cells. Neurology 42 (Suppl 3):252.

35. Bal V, McIndoe A, Denton G, Hudson D, Lombardi G, Lamb J, Lechler R (1990): Antigen presentation by keratinocytes induces tolerance in human T cells. Eur J Immunol 20:1893-1902.

36. Daubener W, Zennati S S, Wernet P, Bilzer T, Fischer HG, Hadding U (1992): Human glioblastoma cell line 86HG39 activates T cells in an antigen specific major histocompatability complex class II-dependent manner. J Neuroimmunol 41: 21-28.

37. Aloisi F, Borsellino G, Samoggia P, Testa V, Chelucci C, Russo G, Peschle C, Levi G (1993): Astrocyte cultures from human embryonic brain: characterization and modulation of surface molecules by inflammatory cytokines. J Neurosci Res 32:494-506.

38. Linsley PS, Brady W, Grosmaire L, Aruffo A, Damle NK, Ledbetter JA (1991): Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA acumulation. J Exp Med 173:721-730.

39. Freedman GJ, Freedman AS, Segil JM, Lee G, Whitman JF, Dadler LM (1989): Selective induction of B7/BB-1 on interferon gamma- stimulated monocytes: a potential mechanism for amplification of T-cell activation through the CD28 pathway. Cell Immunol 137:429-441.

40. Fontana A, Kristensen F, Dubs R, Gemsa D, Weber E (1982): Production of prostaglandin E and interleukin-1 like factor by cultured astrocytes and C6 glioma cells. J Immunol 129:2413-2419.

41. Benveniste EN, Sparacio SM, Norris JG, Grenett HE, Fuller GM (1990): Induction and regulation of interleukin-6 gene expression by rat astrocytes. J Neuroimmunol 30:201-212. 42. Hetier E, Ayala J, Denefle A. (1988): Brain macrophages synthesize interleukin-1 and interleukin-1 mRNA in vitro. J Neurosci Res 21:391-397.

43. Giulian D, Corpuz M (1993): Microglial secretion products and their impact on the nervous system. In Seil FJ (ed.):"Advances in Neurology." New York:Raven Press, Ltd, Vol 59, 315-320.

44. Webb DSA, Shimizu Y, Van Seventer GA, Shaw S, Gerrad TL (1990): LFA-3, CD44, and CD45: physiologic triggers of human monocyte TNF and IL-1 release. Science 249:1295-1297.

TABLE I

Response of Allogeneic and Autologous Mononuclear Cells (MNC) to Mixed Glial Cell Cultures

	Experiment I		Experiment II	
	Auto-MNC	**Allo-MNC**	**Auto-MNC**	**Allo-MNC**
+glia	163(155)	14,355(11,301)	423	16,865
-glia	439	58	197	96

- ()= addition of IFNγ 48 hours prior to addition of MNC's;
- ^{3}H-thymidine uptake of glial cells alone was < 100 cpm.
- Auto= autologous cells; allo= allogeneic cells
- Results represent the mean ^{3}H-thymidine uptake of triplicate wells

TABLE II

Immune Accessory Molecule Expression by Adult Human Microglia and Fetal Human Astrocytes

Marker	Microglia		Astrocyte	
	NS	IFN	NS	IFN
LFA-1	++	++++	-	-
LFA-3	+++	++++	+	+
ICAM-I	++++	++++	++	+++
B7	-	+++	-	-

Results represent the grading of 4 adult human microglial cultures and 4 fetal human astrocyte cultures; 4 coverslips from each culture were graded blindly, 200 cells per coverslip were counted and assessed for staining where (-)= staining absent; (+)=<20% of the cells were positive; (++)= 20-50% of the cells were positive; (+++) 50-80% of the cells were positive; (++++)> 80% of the cells were positive; NS= cells under basal culture conditions; IFN= cells stimulated with IFN gamma for 48 hours prior to staining.

TABLE III

Presentation of Recall Antigens (Candida albicans) to Autologous T Cells by Human Adult-Derived Microglia

	% microglia			
T cells + antigen	**0**	**1%**	**5%**	**10%**
Experiment I	220	507	11,852	13,414
Experiment II	393	11,887	13,363	17,545

% microglia represent ratio of the number of microglia (APC) over the total number of T cells (10^5 cells per well). Results represent the mean ^{3}H-thymidine uptake (cpm) of triplicate wells. Microglia alone <300 cpm.

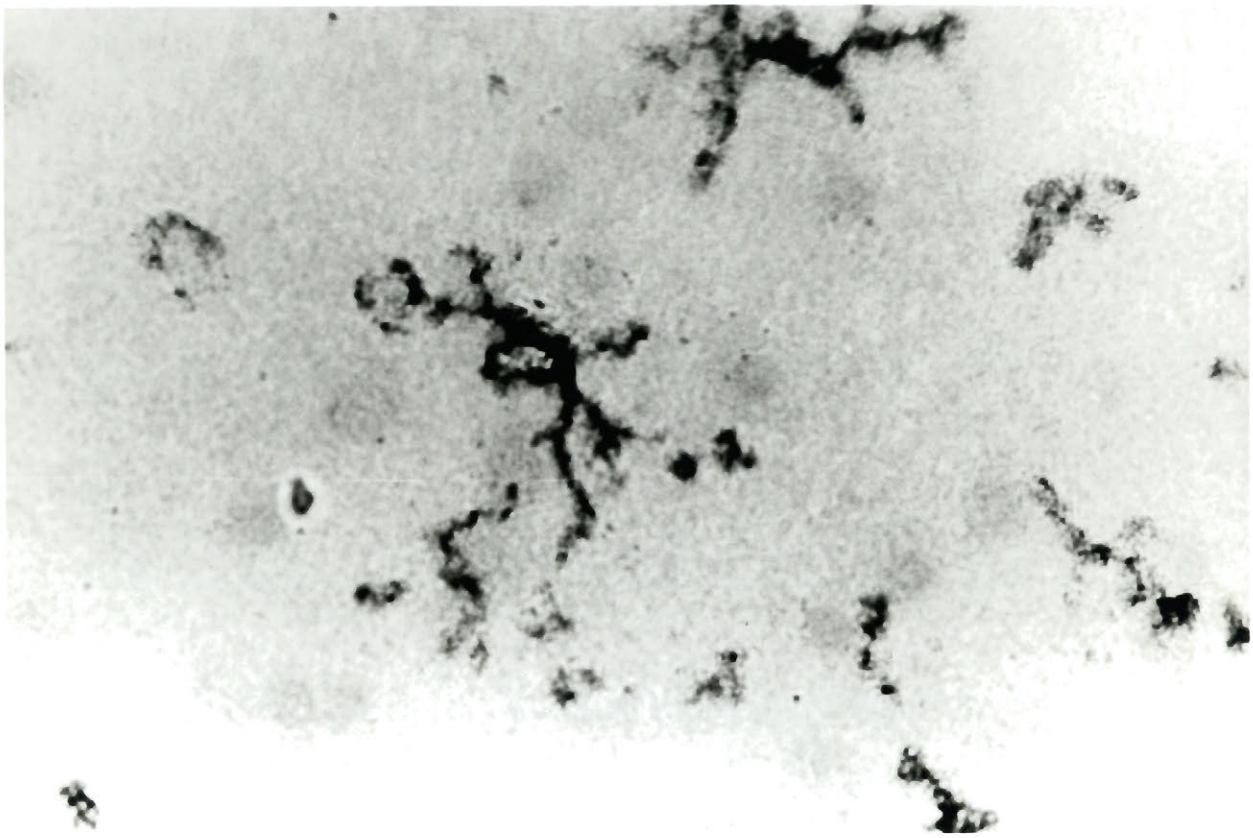
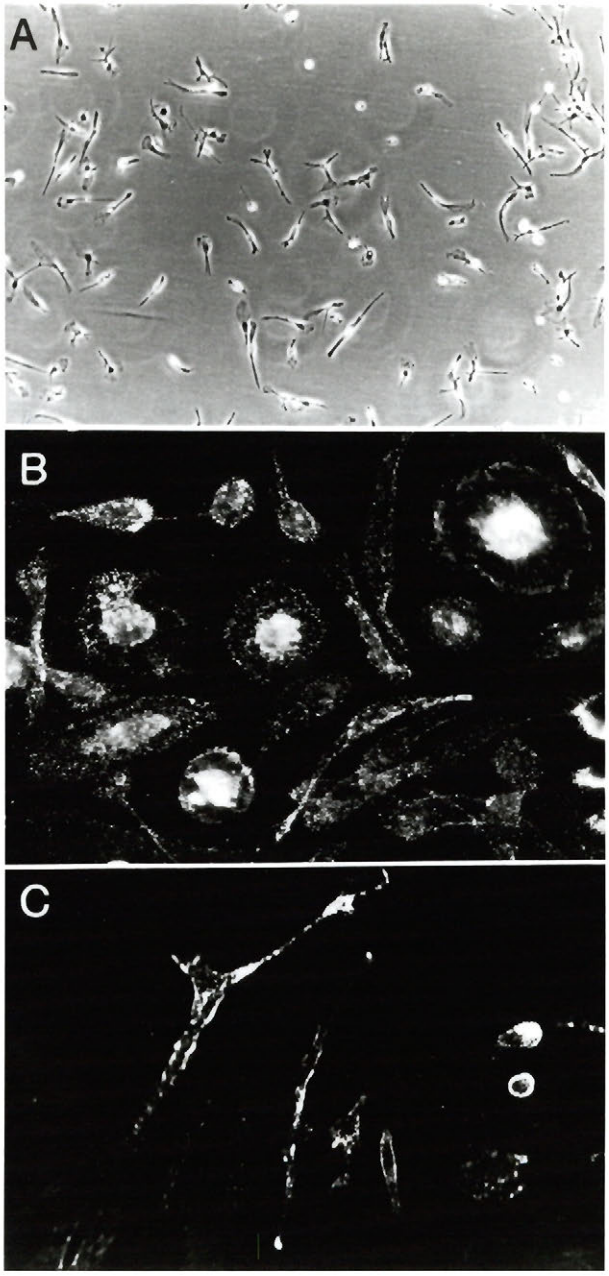


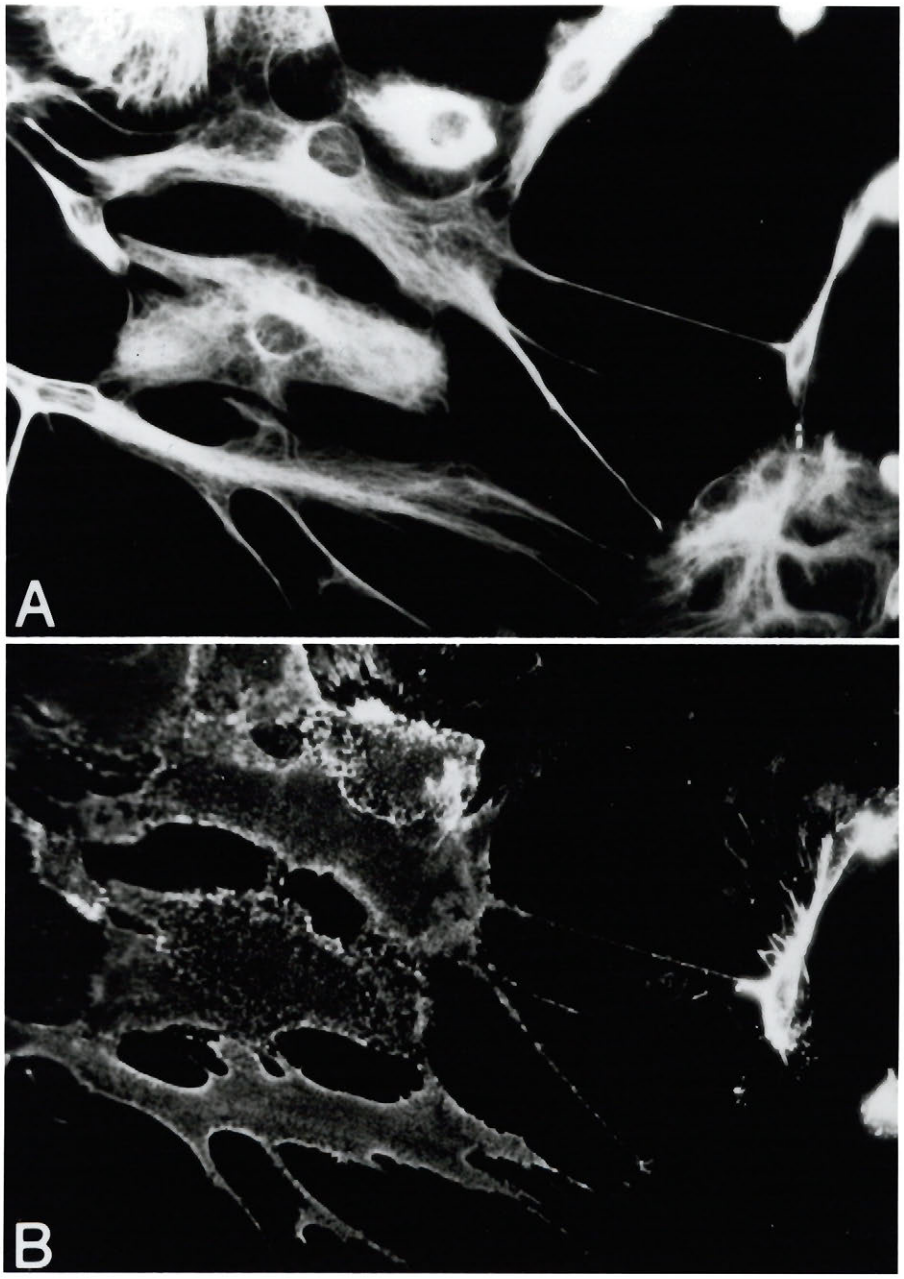
Figure 1. MHC class II expression on parenchymal adult human microglia as assessed using mAb SG465 (400X). Tissue sections were taken from the same specimens used to establish mixed and purified adult human cultures. MHC class II positive cells were Leu M5 positive as determined by immunocytochemistry of adjacent serial sections (data not shown) (400X).

Figure 2. Adult human microglia in vitro (enriched cultures >95% purity) A) Phase contrast microscopy of live adult human microglia maintained in vitro for one week (200X). B) Leu M5 immunoreactivity of enriched microglial culture (800x). C) Basal expression of MHC class II (HLA-DR) on adult human microglia in vitro assessed using mAb D1-12 (800X).


A
B
C

92-1720

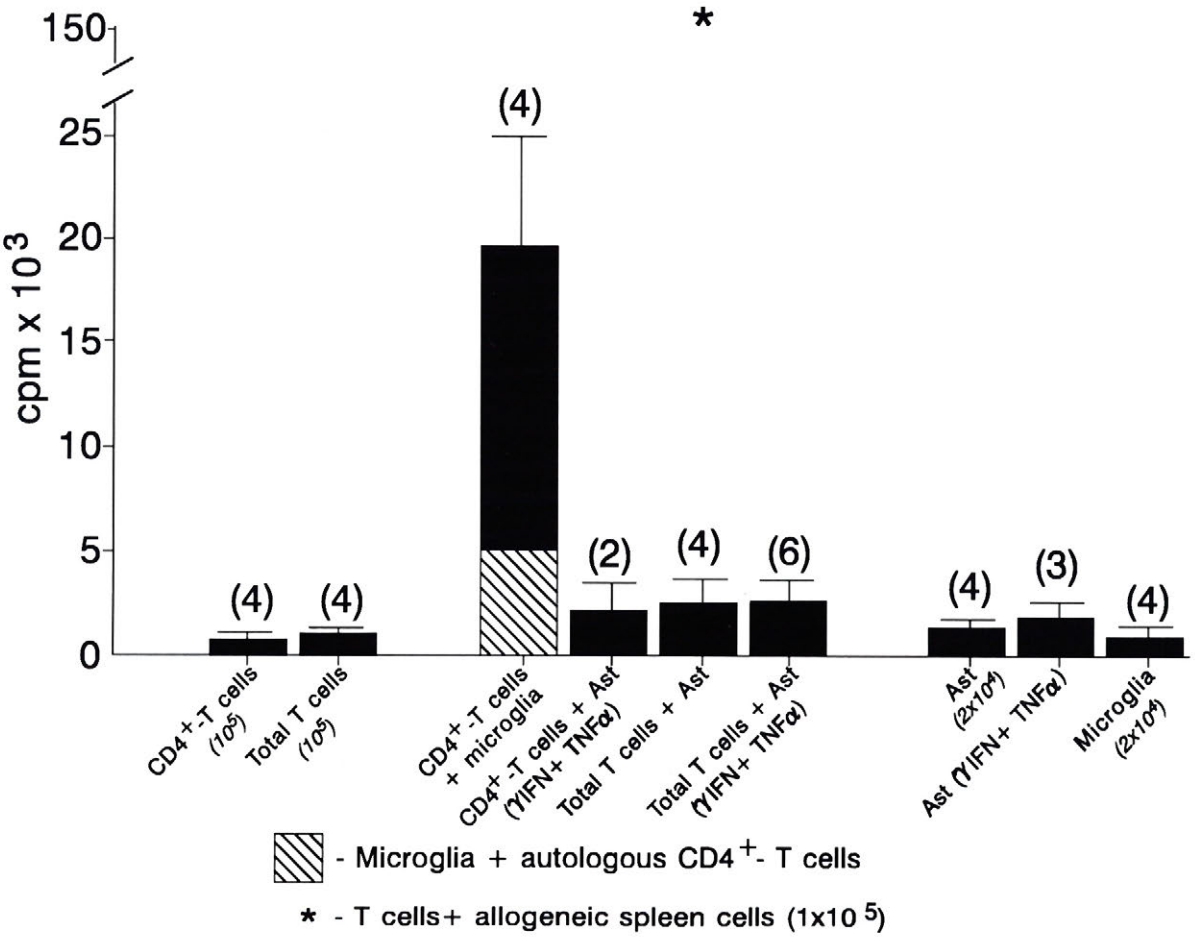
Figure 3. Enriched fetal human astrocytes (>95% purity) treated with IFNγ (100 U/ml) and TNFα (100 U/ml) for 48 hours. A) GFAP immunoreactivity and B) HLA-DR immunoreactivity of the same field (800X).


A
B

92-1360

Figure 4. Stimulation of allogeneic T cells by human microglia and astrocytes. Bars represent counts per minute (CPM) of either 10^5 T cells alone, T cells cocultured with microglia or astrocytes, or microglia and astrocytes ($2X10^4$) alone. For the T cell- glia cocultures, solid bars represent allogeneic T cells, hatched bar represents autologous T cells. Brackets () indicate the number of experiments per data point. Each individual experiment represents means of triplicate wells $\pm$ the standard error of the mean (SEM). CPM of radiated glial cells or $CD4^+$ T cells alone was less than 800 CPM; * = irradiated fetal spleen cells incubated with allogeneic T cells.

Stimulation Of Allogeneic T Cells By Human Microglia And Astrocytes.



92-1369

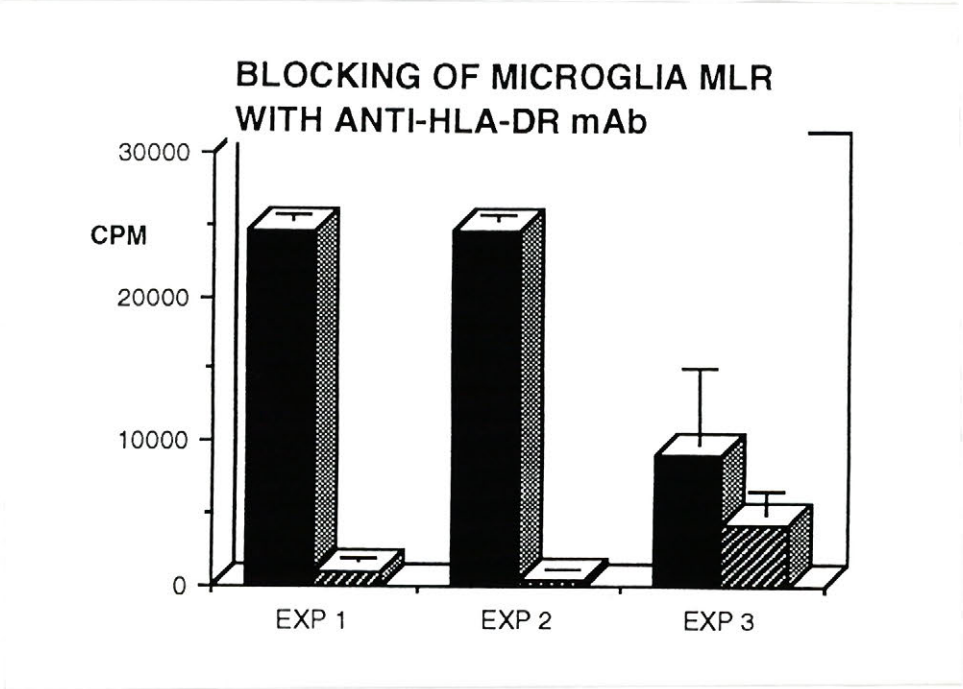


Figure 5. Blocking of microglia stimulated MLR with anti-HLA-DR mAb L243. Solid bars represent CPM (^{3}H-thymidine) of 10^5 $CD4^+$ T cells cocultured with $2X10^4$ adult human microglia. Hatched bars represent CPM of 10^5 $CD4^+$ T cells cocultured with $2X10^4$ microglia pre-incubated with mAb L243 (diluted 1:300) 1 hour prior to the addition of the T cells. Results are expressed as mean CPM of triplicate wells $\pm$ SEM.

Chapter 5

ANTIGEN PRESENTATION BY HUMAN FETAL DERIVED ASTROCYTES: THE COOPERATIVE EFFECT OF MICROGLIA OR THE MICROGLIAL DERIVED CYTOKINE IL-1

Kenneth C. Williams, Nora P. Dooley, Elling Ulvestad, Anders Waage, Manon Blain, Voon Wee Yong, and Jack P. Antel

Preface

Following the observation that microglia, but not astrocytes, could present antigen in a primary mixed lymphocyte reaction, we sought to determine biological differences in the capacity of microglia versus astrocytes to present antigen. In the previous chapter, microglia and astrocytes were compared in terms of immune accessory molecules considered important as secondary signals provided by antigen presenting cells for T cell activation. In this chapter, employing RT-PCR and cytokine bioassays, cytokine message and production by microglia and astrocytes was compared. Major points demonstrated in this chapter include:

1. Astrocytes could not present antigen in primary mixed lymphocyte reaction to immediately ex-vivo $CD4^+$ T cells. With the addition of human rIL-2, astrocytes were able to support the proliferation of T cells suggesting that while astrocytes were not able to stimulate the T cells to proliferation, astrocytes were able to stimulate the $CD4^+$ T cells to become IL-2 responsive.

2. Microglia had cDNA corresponding to mRNA for IL-1α, IL-6, and TNFα, while astrocyte had detectable basal message for IL-6. Cytokine bioassays confirmed RT-PCR results.

3. Using a combination of human rIL-1α, IL-6, and TNFα added to astrocyte

$CD4^+$ T cell co-cultures, it was found that IL-1α alone could reverse the inability of astrocytes to present in the mixed lymphocyte reaction. The reversal response was demonstrated using as low as 1 U/ml of human rIL-1.

4. In addition to IL-1α, microglia at non-T cell stimulating concentrations of 1-$5x10^3$ cells per well with astrocyte T cell co-cultures could also result in the cell stimulation.

Abstract

We have previously observed using a MLR system that adult human derived microglia can function as antigen presenting cells for immediately ex-vivo $CD4^+$ T cell responses whereas astrocytes could not. We have now found that fetal human astrocytes can support $CD4^+$ T cell proliferation in the presence of exogenous IL-2, and the continued proliferation of $CD4^+$ T cells previously sensitized to sister astrocyte cultures. To examine the cellular basis for the inability of human astrocytes to function as APC in the primary MLR, astrocyte and microglial enriched populations were established from human embryonic and adult brain respectively and analyzed for their ability to synthesize cytokines potentially relevant as accessory signals in the MLR. Microglia had transcript as determined by the reverse transcriptase-polymerase chain reaction (RT-PCR) and protein as determined by bioassay for IL-1α, IL-6, and TNFα. Human fetal astrocytes had transcript for IL-6 but not for IL-1α or TNFα under basal culture conditions and following stimulation with IFN gamma. The addition of human rIL-1 from 1-50 U/ml, could reverse the inability of astrocytes to present antigen in the primary MLR. In addition, adult human microglia, seeded into the non-priming astrocyte: $CD4^+$ T cell cocultures at non-priming doses of 1,000 to 5,000 cells could also reverse the inability of astrocytes to present antigen in the MLR. These studies suggest that although, in vitro, astrocytes absent of microglia cannot present antigen to immediately ex-vivo blood derived $CD4^+$ T cells in the MLR, in situ, with the cooperative help of microglia derived cytokine or accessory surface molecules, astrocytes may be able to function as APC.

INTRODUCTION

Perivascular lymphocyte infiltrates, reactive astrocytes, and activated microglia are hallmarks of the cellular changes seen in active lesions in the chronic central nervous system (CNS)[3] demyelinating disease multiple sclerosis (MS). The majority of the perivascular lymphocytes in MS lesions are T cells possibly

representing a restricted cellular immune response which might be specific for CNS antigens including myelin basic protein (1). While multiple immune effector mechanisms probably mediate tissue injury in MS, entry of T cells into the CNS parenchyma is one of the early indicators of disease pathology. Whether initial or subsequent sensitization to CNS antigens occurs in the periphery and/or in the CNS is unresolved.

Activated T cell blasts, regardless of MHC class II restriction or antigen specificity, have an enhanced capacity to cross the blood brain barrier and to enter into the CNS (2). However once inflammation is initiated in the CNS, the passive recruitment of non-activated T cells could occur. Whether T cells migrating or recruited to the CNS are subsequently activated or restimulated to continue to proliferate, or instead become anergic or apoptotic (3,4) may depend both upon the brain microenviroment (5) and putative CNS antigen presenting cells (6).

Astrocytes and microglia have been implicated as resident CNS cells capable of presenting antigen to MHC class II restricted $CD4^+$ T cells. While astrocytes have been reported to express MHC class II antigens in CNS disease (7-9), microglia are considered to be the major cell type expressing class II antigens in "normal" individuals (10-13) and in MS lesions (14,15).

Rodent astrocytes (16-18) and microglia (19,20) can both express MHC class II antigens in vitro and function as APC capable of supporting the continued proliferation of Ag-specific $CD4^+$ T cell lines. We have recently shown that adult human brain-derived microglia can present recall antigen to freshly derived autologous $CD4^+$ T cells (21) and present antigen to immediately ex-vivo blood derived allogeneic $CD4^+$ T cells in a MLR. Consistent with previous observations using fetal rodent astrocytes (22), we found that fetal human astrocytes expressing MHC class II antigens were unable to initiate a similar $CD4^+$ T cell response (23).

To define a cellular basis for differences in the antigen presentation capacity between microglia and astrocytes, we have previously documented that in vitro both cell types express MHC class II, LFA-3, and ICAM-1 molecules constitutively, and that microglia additionally express LFA-1 and B7/BB-1, particularly following IFN

gamma stimulation (23). We have now compared human microglia and astrocytes with regard to selected cytokine gene expression and production as a means to delineate the basis for the inability of human fetal astrocytes to initiate an immune response. Using the reverse transcriptase- polymerase chain reaction (RT-PCR) and cytokine bioassays, we found that human adult microglia expressed mRNA and synthesized protein for IL-1α, IL-6, and TNFα, whereas human fetal astrocytes had message and protein for IL-6, but not IL-1α or TNFα. Addition of human rIL-1α, as low as 1 U/ml, reversed the inability of astrocytes to present antigen in a MLR to freshly isolated human peripheral blood derived $CD4^+$ T cells. Addition of as few as 1,000 to 5,000 microglial cells into the non-priming astrocyte MLR also resulted in a significant $CD4^+$ T cell response. These data suggest that purified human astrocytes, without contaminating microglia, are unable to present antigen and activate freshly derived $CD4^+$ T cells in vitro. In the CNS, however, astrocytes may be able to present antigen and initiate immune responses with the cooperative effect of soluble microglial cytokines or microglial accessory surface molecules.

MATERIALS AND METHODS

Source of glial cells - Adult human brain tissue served as the source of microglia and was obtained from surgical resections, carried out to ameliorate non-tumor related intractable epilepsy (N=7 patients) (ages 18-45). Tissues used were derived from regions requiring resection to reach the precise epileptic focus, and were distant from the main electrically active site. Fetal human CNS tissues served as the source of astrocytes and were prepared from 8-12 week old specimens (N=8) following Medical Research Council of Canada approved guidelines.

Glial cell preparations

Adult - Our method for preparing adult human glial cell cultures has been previously described (21,24,25). Briefly, tissues were treated with 0.25% trypsin and DNase (50 μg/ml) followed by a percoll gradient centrifugation (Pharmacia LKB Biotechnology, Uppsala, Sweden) at 15,000 RPM for 30 min. A mixed,

dissociated glial cell suspension was then suspended in culture medium consisting of Eagle's MEM supplemented with 5% FCS, 0.1% glucose, and 20 μg/ml gentamicin and seeded into 25 cm^2 tissue culture flasks (Nunc, Burlington, Ontario). The following day, oligodendrocytes remained floating and were removed. Remaining adherent cells consisting of astrocytes and microglia were allowed to develop morphologically for 7 days. At this time the less adherent astrocytes were then floated off by rotary shaking for 5 hours at 150 rpm. Microglia were detached by incubation of cells using 0.25% trypsin and DNase (50μg/ml) at 37 °C for 15 min, and seeded into either 96 well microtitre plates or onto Aclar fluorocarbon coated coverslips (Dr. S Kim, Vancouver, British Columbia) previously coated with 10 μg/ml poly-L-lysine.

Fetal - Fetal cultures were prepared by carefully stripping CNS material of meninges and blood vessels, mechanically dissociating the tissue with scalpel blades followed by treatment with trypsin (0.25%) and DNase (50 μg/ml) at 37°C for 45 min. Dissociated tissue was then passed through a 130 μm nylon mesh, washed 2X in PBS and cells were plated directly onto poly-L-lysine coated plastic culture dishes in culture medium (Yong et al., 1991). Cultures consisting of astrocytes, neurons, and sparse microglia, were split when confluent (approximately every 2 weeks). Experiments were conducted after the second or third cell passage when fetal neurons and microglia were no longer apparent in culture. Similar to microglia, astrocytes were seeded into 96 well plates at a density of $2x10^4$ cells per well.

Purity of microglia and astrocyte cultures was determined by immunostaining with anti-CD11c (Leu-M5, 1:10) (Beckton-Dickinson, San Jose, CA) or rabbit anti-GFAP Ab (1:100) (Dako, Westchester, PA) followed by goat anti-mouse Ig-Rh (1:150) (Cappel, Lexington, MA) or goat anti-rabbit Ig-FITC (1:150) (Cappel). Prior to staining for GFAP, cells on coverslips were fixed with acid alcohol (5% glacial acetic acid:95% absolute ethanol, v/v). Proportions of immunostained cells were assessed by counting using a fluorescence microscope, or

by FACScan analysis (Beckton Dickson, Mountainview, CA).

$CD4^+$ T-lymphocyte Preparations

Mononuclear cells (MNC) were isolated by Ficoll-Hypaque density centrifugation from heparinized peripheral blood samples obtained from young adult volunteers. Total T cells were isolated by rosetting MNC with S-(2-aminoethyl) isothiouronium bromide hydrobromide (AET)- treated sheep red blood cells (23). The rosette positive T cells (E^+) were further purified as described below. Mononuclear cells that did not rosette (E^-), consisting of B cells and monocytes, were irradiated (2,500 rad) and used in some MLR assays as controls. $CD4^+$ T cell populations were prepared by incubating total T cells with anti-CD8 monoclonal antibodies (prepared from OKT8 hybridoma obtained from ATCC, Rockville, MD), followed by the addition of rabbit complement (Cedarlain Laboratories, Hornby, Ontario). Cells were cultured in medium consisting of RPMI supplemented with 5% human AB serum (Pel-Frez, Brown Deer, WI) 2.5 μg/ml penicillin, and 2.5 μg/ml streptomycin. The purity of the $CD4^+$ T cells, assessed by immunostaining with fluorescein-labelled anti-CD8 mAb (Leu2) (Beckton-Dickinson) and phycoerythrein conjugated anti-CD4 mAb (Leu 3a) (Beckton-Dickinson), and analyzed by FACScan (Beckton-Dickinson), was >96%.

Cytokine analysis

Using the reverse transcriptase polymerase chain reaction (RT-PCR) cytokine gene expression was assessed using enriched microglia and astrocyte cultures, and brain biopsy specimens (taken from the same material used to establish glial cell cultures) that had been snap frozen in liquid nitrogen. Some astrocyte cultures were preincubated with IFN gamma for 24 hours prior to RNA extraction. Total RNA was isolated using the guanidinium isothiocyanate-phenol-chloroform method (26). Contaminating DNA was removed by treatment with 1U RNase free DNase (RQ1 DNase, Promega) for 30 minutes at 37°C in 40mM Tris HCl pH 7.9, 10mM NaCl, and 6mM $MgCl_2$. The RNA was then re-extracted with

phenol, ethanol precipitated, and stored at -20°C.

RNA (0.2-0.5 μg) was reverse-transcribed and amplified in a single-step process as described by Singer-Sam et al. (27) with the following modifications: 5 U AMV reverse transcriptase (Gibco), 40 U RNA-guard (Pharmacia, Uppsala, Sweden), 12.5 U TAQ DNA polymerase (Gibco), 50μM dNTPs (Pharmacia), 50pM primers (Sheldon Biotechnology, Montreal, PQ), and 0.5μCi of alpha-^{32}P-dATP (Amersham, Arlington Heights, IL). The total reaction volume of 50 μl was then overlaid with 100 μl of heavy mineral oil. Samples were placed in a thermocycler (Cetus, Perkin Elmer, Norwalk, CT) for 15 mins at 50°C, followed by 20 cycles of 94°C for 1 min, 55°C for 2 mins, and 72°C for 2.5 mins. Following amplification, 25μl of each sample was electrophoresed on a 10% non-denaturing polyacrylamide gel which was then dried under vacuum and visualized by autoradiography.

The sequence of the primers used were previously determined by others to be specific for the cytokine of interest. The oligonucleotide sequences are as follows: IL-1α forward (F) 5'-GTCTCTGAATCAGAAATCCTT and reverse (R) 5'-CATGTCAAATTTCACTGCTTCATCC (28), IL-6 F 5'CCAAGAATCTAGATGCAATAAA and R 5'-GCCAATTAACAACAACAATCTG (29), TNF F 5'-CCCTCAAGCTGAGGGGCAGCTCCAG and R 5'-GGGCAATGATCCCAAAGTAGGGGGGACCTG (28). Primers specific for glyceraldehyde-3-phosphate dehydrogenase (GADPH) were also employed as controls for RNA loading. The sequence for this set of primers is as follows: F 5'-CCATGGAGAAGGCTGGGG and R 5'-CAAAGTTGTCATGGATGATGACC (30).

Cytokine bioassays

Assay for IL-1 and IL-2. IL-1 was determined in a two-stage assay by the IL-1 responsive mouse T-cell line EL-4 6.1 clone NOB-1 (31) and the IL-2 dependent mouse T-cell line HT-2 (32). The EL-4 cells produce IL-2 in response to IL-1

stimulation, and the IL-2 production was measured by the proliferation of the IL-2 dependent HT-2 cells. Serial dilutions of samples were added in duplicate to flat-bottom 96-well microtiter plates, and the volume adjusted with medium to 100 µl/well. The medium utilized was RPMI 1640 supplemented with 0.1 mg/ml L-glutamine, 40 µg/ml gentamicin, 10% FCS and 25 µM 2-mercaptoethanol. The EL-4 cells were washed once in Hank's balanced salts (HBSS) (Gibco, Paisley, UK), resuspended in medium and incubated at $2X10^5$ cells/well producing a final volume of 200 µl per well. After 24 hours incubation, 100 µl of supernatant was transferred from each well into a replicate microtiter plate. The HT-2 cells were washed three times in HBSS, resuspended in medium and distributed to the microtiter plates at a concentration of 0.15×10^5 cells/well giving a final volume of 200 µl/well. After 20 hours of incubation the cell growth was measured by the colorimetric MTT assay. Human rIL-1ß (Glaxo, Geneva, Switzerland) was included as a standard. The detection limit of the assay was 15 pg rIL-1/ml supernatant.

Assay for IL-6. IL-6 was determined by the IL-6 dependent mouse hybridoma cell line B13.29 clone B9 (33). The cells were incubated in the presence of serially diluted samples and growth measured after 72 hours using the MTT colorimetric assay. Human rIL-6 (Dr. L. Aarden, University of Amsterdam, The Netherlands) was used as standard. The detection limit of the assay was 15 pg/ml.

Assay for TNF. TNF was determined by its cytotoxic effect on the mouse fibrosarcoma cell line WEHI 164 clone 13 (34). Cells were incubated with serial dilutions of test supernatants and viability was measured after 20 hours by a colorimetric assay based on a tetrazolium salt as previously described (34,35). Human recombinant TNF (Genetech, San Francisco, CA) was used as standard. The detection limit of the assay was 3 pg rTNF/ml supernatant.

Functional $CD4^+$ T cell assays

Primary MLR - For these studies $1x10^5$ $CD4^+$ T cells from at least two different donors per experiment were placed into individual wells of flat-bottom microtitre plates, in which fetal astrocytes had been previously seeded at confluence ($2x10^4$ cells per well). Astrocytes were irradiated in the 96 well plates with 2,500 rads (AECL Gamma Cell 1000 Irradiator) prior to the addition of $CD4^+$ T cells. Fetal astrocytes were used under basal culture conditions and following stimulation with human rIFN gamma (100 U/ml) (Boehringer Mannheim, Germany) and TNFα (100 U/ML) (Genzyme, Boston, MA) 48 hrs prior to lymphocyte coculture. Culture wells were washed extensively prior to all T cell assays. The mixed lymphocyte reaction was carried out for 5 days. To assess $CD4^+$ T cell proliferation, the cells were pulsed for 5 hours with [^{3}H]-TdR (1 uCi per well) (ICN Flow Laboratories, Mississauga, Ontario), harvested, and the counts determined using a beta liquid scintillation counter (LKB, Fisher, Montreal). Results are expressed as mean counts per minute (CPM) of triplicate wells.

Initial titration studies had been undertaken to determine at which seeding concentrations microglia and astrocytes stimulate $CD4^+$ T cells in an allogeneic MLR. For these studies irradiated microglia (2,500 rads) and astrocytes, and non-rosetting mononuclear cells (E^-), consisting of monocytes and B lymphocytes, were seeded into 96 well flat bottom microtitre plates at seeding densities ranging from 10^2 to $1x10^5$ cells per well. To these, $1x10^5$ allogeneic $CD4^+$ T cells (from 2 donors per experiment) were added as described below.

Secondary MLR- For these studies $1x10^7$ freshly isolated $CD4^+$ T cells were coincubated with confluent bulk astrocyte cultures in 25 cm^2 flasks with human rIL-2 (50 U/ml) (Genzyme, Boston, MA). Cells were incubated for 5 days at which time lymphocytes were recovered and then introduced back into a MLR with sister fetal astrocyte cultures as described above.

Cytokine and microglia supplemented MLR

In some experiments human rIL-1α (1-50 U/ml) (Genzyme), rIL-2 (50 U/ml) (Genzyme) rIL-6 (50 U/ml) (UBI, Lake Placid, NY) and rTNFα (50 U/ml) (UBI) was added to the glial-CD4$^+$ T cell cocultures at the beginning of the assay. In subsequent experiments to neutralize IL-1α activity, an IL-1 receptor antagonist (IL-1RA) (Pepro Tech, Rocky Hill, NJ) (5 ng/ml) was added concurrently with rIL-1α. To study the ability of microglia to reverse the non-priming response, microglia, at non-priming doses, were seeded into the MLR at either 1,000 or 5,000 cells per well with the addition of CD4$^+$ T cells.

RESULTS

Glial cell cultures

As previously reported (21,23) enriched human microglial cultures in excess of 95% Leu-M5$^+$ cells were established (Figure 1). Additionally, microglia were Leu-M3$^-$ and non-specific esterase (NSE) negative, distinguishing them from peripheral blood monocytes which are Leu-M3$^+$ and NSE$^+$ (21). The remaining contaminating cells in microglia cultures were GFAP positive astrocytes. Fetal astrocyte cultures were similarly established with a high degree of purity (>95%) with contaminating cells being neuronal in origin (Fig 2a and b). Adult human astrocytes could not be purified beyond 70% purity (24) and therefore could not be used in this study which required more highly enriched cultures.

Cytokine gene and protein

Using RT-PCR, cDNA corresponding to IL-1α, IL-6, and TNFα were amplified from total microglial RNA isolated under basal tissue culture conditions, whereas cDNA corresponding to IL-6 were amplified from astrocyte RNA (Figure 3). Human fetal astrocyte cultures, even after stimulation for 48 hours with IFN gamma, did not express detectable message for IL-1α or TNFα. The exclusive positive reaction product for IL-1α and TNFα by microglia suggest that the reaction product was not the result of contamination by astrocytes in the microglial cultures.

Similarly, the lack of IL-1α and TNFα message by fetal astrocytes would argue against microglial contamination in the fetal astrocyte cultures. Cytokine bioassays confirmed that microglia produce low levels of IL-1 (6.7 pg/ml $\pm$ .7 pg/ml) under basal culture conditions that increased following incubation with IFN gamma (72.2 pg/ml $\pm$ 8.9 pg/ml). Microglia under basal culture conditions produced IL-6 (359 pg/ml $\pm$ 50 pg/ml) and TNF (221 pg/ml $\pm$ 28 pg/ml) that increased following IFN gamma stimulation (IL-6, 1401 pg/ml $\pm$ 279) (TNF, 1601 pg/ml $\pm$ 45) (Figure 4). Human fetal astrocytes produced low IL-6 under basal culture conditions (30 pg/ml $\pm$ 12 pg/ml) that increased following IFN gamma stimulation (191 pg/ml $\pm$ 31 pg/ml). There were no detectable levels of IL-1 or TNF by human fetal astrocytes under basal culture conditions or following stimulation (Figure 4).

Primary MLR

As seen in Figure 5, titration studies showed that human derived microglia were able to stimulate immediately ex-vivo $CD4^+$ T cells at seeding densities from 2-4x10^4 cells per well. E^- populations stimulated $CD4^+$ T cells at E^- concentrations starting at 2 X 10^4 cells through 5 X 10^4 cells per well. Human fetal astrocytes under basal culture conditions or following incubation with IFN gamma (100 U/ml, for 48 hr), did not stimulate freshly isolated $CD4^+$ T lymphocytes in the allogenic MLR.

To test whether astrocytes cocultured with fresh $CD4^+$ T cells provided any level of stimulation, experiments were performed where human rIL-2 was added to astrocyte: $CD4^+$ T cell cocultures. Figure 6 shows that the addition of human rIL-2 to astrocyte- $CD4^+$ T cell cocultures resulted in $CD4^+$ T cell proliferation whereas the addition of rIL-2 to $CD4^+$ T cells alone did not; indicating that astrocytes could stimulate $CD4^+$ T cells to become IL-2 responsive. The magnitude of the $CD4^+$ T cell proliferative response following addition of human rIL-2 was greater when astrocyte cultures had been pre-stimulated with IFN gamma (100 U/ml, 48 hr) prior to the addition of $CD4^+$ T cells (Figure 6). When freshly isolated $CD4^+$ T lymphocytes sensitized to fetal astrocyte cultures <u>in vitro</u>, in the

presence of rIL-2 for 5 days, were recovered, washed, and reintroduced to sister astrocyte cultures (2° MLR), a significant proliferative response was observed (Figure 7). This response, indicating the ability of astrocytes to support the proliferation of previously activated $CD4^+$ T cells, was not demonstrated using fresh $CD4^+$ T cells from the same donor in a primary MLR. The ability of astrocytes to support the proliferation of previously activated $CD4^+$ T cells was partially inhibited by an anti-HLA-DR blocking antibody L243. (Figure 7).

RT-PCR and bioassay data demonstrated that microglia alone had transcript for IL-1α and TNFα under basal culture conditions and following activation, and corresponding cytokine production. Using human r-cytokines, we investigated whether IL-1, IL-6, or TNF could provide a necessary secondary signal for astrocytes to present antigen in the MLR. As shown in Table 1, the addition of human rIL-1α (10-50 U/ml) to astrocyte:$CD4^+$ T cell cultures resulted in significant T cell proliferation. In subsequent experiments, we were able to demonstrate $CD4^+$ T cell proliferation using as low as 1 U/ml rIL-1α (Figure 8); the maximal response was consistently observed following the addition of 10 U/ml human rIL-1α (Table 1, Figure 8). Human rIL-6 and rTNFα, used either separately or in conjunction with IL-1α, did not augment $CD4^+$ T cell proliferation beyond basal control levels or IL-1α induced levels respectively (data not shown). Co-incubation of fetal astrocyte: $CD4^+$ T cell cocultures with human r-IL-1α (50 U/ml) and the IL-1 receptor antagonist (IL-1RA) inhibited the IL-1 priming effect (26,715 $\pm$ 7,375 cpm vs. 3,258 $\pm$ 687 cpm (n=4 experiments).

To investigate whether microglia could provide a secondary signal to induce $CD4^+$ T cell proliferation in the presence of non-stimulating astrocytes, microglia were seeded into the MLR at 1,000 or 5,000 cells per well; concentrations at which microglia alone did not induce proliferation in the MLR (Figure 5). As shown in Figure 9, as few as 1,000 microglia seeded into the non-priming MLR could effectively correct the inability of astrocytes to induce $CD4^+$ T cell proliferation to the same amplitude or greater than the rIL-1α control. Similar to the response with human rIL-2, the microglial induced proliferation resulted in greater $CD4^+$ T cell

proliferation when fetal astrocytes were stimulated with IFN gamma prior to the addition of microglia and $CD4^+$ T cells. The addition of the IL-1RA (5ng/ml) to astrocyte/microglia/T cell cocultures did not result in significant reduction of the proliferative response (N=4) (data not shown).

DISCUSSION

This study addresses whether and under what conditions astrocytes can function as APC to initiate or perpetuate a CNS immune response. The CNS has been regarded as an immunologically privileged site having minimal numbers of cells expressing MHC class II antigens (36) and being inaccessible to circulating lymphocytes (37). More recent studies have demonstrated that in the CNS immune surveillance does occur (2,38), that MHC class II antigens are expressed in normal and diseased individuals (11,12,15,39) and that an active immune-regulation might be exerted (40). Based upon initial observations of MHC class II expression in human inflammatory CNS disease and in EAE, astrocytes were the focus of early CNS antigen presentation studies. More recent evidence suggests that if MHC class II antigens are expressed on astrocytes, the level of Class II expression is marginal or occurs late in the disease, after significant inflammation has occurred (7,15,39,41,42). Additionally, <u>in vitro</u> models studying antigen presentation by rodent astrocytes have mainly utilized T cell lines which are by definition previously activated cells and more accurately represent secondary T cell responses (17,18). In our study, fetal human astrocytes, in the absence of added cytokines, were unable to present antigen in the primary MLR. Astrocytes were able to support $CD4^+$ T cell proliferation in the presence of exogenous IL-2, suggesting that the astrocytes were able to stimulate lymphocytes to express IL-2 receptors and to become IL-2 responsive, but not to produce IL-2 and proliferate (43). Astrocytes were also able to support the proliferation of $CD4^+$ T cells previously sensitized to sister astrocyte cultures in a 2° MLR. We have previously demonstrated that irradiated fetal human spleen cells obtained from the same donor material as the astrocytes, are potent stimulators in the MLR, ruling out the

inability of astrocytes to prime based on embryologic development (23).

In addition to the requirement of class II expression for the APC to present antigen to MHC class II restricted $CD4^+$ T cells, additional signals thought to be important in T cell activation include the molecules ICAM-1, LFA-3, B7 (44,45,46) and accessory signals including IL-1α, IL-6 and TNFα (47,48). We have previously demonstrated that fetal human astrocytes and adult human microglia could express similar adhesion molecules including MHC class II, LFA-3, and ICAM-1, though microglia consistently expressed higher levels of these molecules. Microglia also were positive for LFA-1 and B7-BB1, the latter being more markedly expressed following induction with IFN gamma (23).

In the current report we further investigated the inability of fetal human astrocytes, a non-haematopoietic, MHC class II expressing cell, to stimulate $CD4^+$ T cells in a MLR; in particular, we compared cytokine production by astrocytes and microglia. Our data for adult human microglia cytokine production is consistent with previous reports for rodent (49-51) and human (52-54) fetal microglial cultures. Cytokine production by astrocytes is not resolved and remains controversial (52). Similar to previous reports using fetal human (52-54) and rodent derived astrocytes (49) we did not detect IL-1α message or protein in our purified fetal astrocyte cultures under basal culture conditions or following IFN gamma stimulation. Others have, however, reported the synthesis of IL-1 by rodent astrocytes or astrocyte cell lines (55-57). Similarly, there are reports of TNFα mRNA and protein production by human (53) and rodent astrocytes following viral infection or induction with IL-1ß or LPS (58,59). Our data indicating a lack of IL-1α production by human fetal astrocytes is further supported by our functional data showing that recombinant human IL-1α could reverse the inability of astrocytes to stimulate T cells in the MLR. IL-1 has been shown to be elevated in both a rodent CNS injury model (51) and in MS serum and CSF (60,61). The reversal of the inability of astrocytes to present in the MLR by exogenous IL-1 was blocked using an IL-1 RA which has been shown to abrogate symptoms in animal models of autoimmune disease (62). The inability of the IL-

1RA to inhibit T cell proliferation when microglia were seeded with astrocyte cultures might be due to microglia phagocytosis of the receptor antagonist; alternatively these data might indicate the importance of additional microglial signals including B7/BB-1 or ICAM-1 (44). We have not ruled out that the IL-1 effect might be acting directly on the astrocytes (53)

IL-1 has been previously demonstrated to be a necessary secondary signal provided by a variety of APC including macrophages (63,64) monocytes (65) and B-cells (66), to initiate or perpetuate T cell activation. IL-1 has also been shown to function as an autocrine cytokine for T cell proliferation (67), to be essential for TH2 costimulation (68,69) and to be involved in the priming response by dendritic cells (70,71). Since we found that both microglia and astrocytes produce IL-6, we cannot rule out the possibility that the IL-1α response may be functioning in cooperation with astrocyte produced IL-6 (72).

The type of cells that can stimulate $CD4^+$ T cell proliferation in a MLR remains controversial. A variety of class II expressing, haematopoietic cells have been reported to functions as APC. These cells include B-cells, endothelial cells, dendritic cells, and macrophages (43). T cell contact with MHC class II antigens on non-haematopoietic cells such as astrocytes, usually results in $CD4^+$ T cell tolerance and not T cell proliferation (73). In our assay system, microglia at very low cell numbers, were able to reverse the inability of astrocytes to prime. Recently it has been shown that the ligation of CD28 on T cells by B7 can inhibit or reverse the induction of T cell anergy (74). This might provide an alternative mechanism of microglial stimulation in our assay system where B7/BB-1 expression on microglia might function to inhibit the induction of anergy by astrocytes.

In our assay system, human fetal astrocytes were not able to present antigen in the MLR, but could stimulate $CD4^+$ T cells following the addition rIL-1α as low as 1 U/ml or by the addition of a small number of microglia. Our data raise the interesting possibility that astrocytes in vivo, with the help of an accessory cell providing either a soluble secondary signal or the surface expression of accessory molecules, might effectively stimulate T cell proliferation, thereby initiating or

perpetuating a CNS immune event.

References

1. Oksenberg J.R., M.A. Panzara, A.B. Begovich, D. Mitchell, H.A. Erlich, R.S. Murray, R. Shimonkevitz, M. Sherritt, J. Rothbard, C.C.A. Bernard, and L. Steinman. 1993. Selection for T-cell receptor Vß-Dß-Jß gene rearrangements with specificity for a myelin basic protein peptide in brain lesions of multiple sclerosis. Nature. 362:68.

2. Hickey W.F., B.L. Hsu and H. Kimura. 1991. T-lymphocyte entry into the central nervous system. J Neurosci Res. 28:254.

3. Pender M.P., P.A. McCombe, G. Yoong, and K.B. Nguyen. 1992. Apoptosis of αß T lymphocytes in the nervous system in experimental autoimmune encephalomyelitis: its possible implications for recovery and acquired tolerance. J Autoimmun 5:401.

4. Ohmori K., Y. Hong, M. Fujiwara, and Y. Matsumoto. 1992. In situ demonstration of proliferating cells in rat central nervous system during experimental autoimmune encephalomyelitis. Evidence suggesting that most infiltrating T cells do not proliferate in the target organ. Lab Invest. 66:54.

5. Sloan D.J., M.J. Wood, and H.M. Charlton. 1992. Leucocyte recruitment and inflammation in the CNS, Trends Neurosci 15:276.

6. Wekerle H., C. Linnington, H. Lassmann, R. Meyermann. 1986. Cellular immune reactivity within the CNS. Trends. Neurosci. 9:271.

7. Traugott U., and C.S. Raine. 1985. Multiple sclerosis. Evidence for antigen presentation in situ by endothelial cell and astrocytes. J Neurol Sci. 69:365

8. Traugott U., L.L. Scheinberg, and C.S. Raine. 1985. On the presence of Ia-positive endothelial cells and astrocytes in multiple sclerosis lesions and its relevance to antigen presentation. J Neuroimmunol 8:1.

9. Lee S.C., G.R.W. Moore, G. Golenwsky, and C.S. Raine. 1990. Multiple sclerosis: a role for astroglia in active demyelination suggested by class II MHC expression and ultrastructural study. J Neuropath Exp Neurol 49:122.

10. Mattiace L.A., P. Davies, and D.W. Dickson. 1990. Detection of HLA-DR on microglia in the human brain is a function of both clinical and technical factors. Am J Pathol. 136:1101.

11. Graeber M.B., W.J. Streit, D.B. Buringer, D.L. Sparks, and G.W. Kreutzberg. 1992. Ultrastructural location of major histocompatibility complex (MHC) class II positive perivascular cells in histologically normal human brain. J Neuropath Exp Neurol. 51:303.

12. Sasaki A., and Y. Nakazato. 1992. The identity of cells expressing MHC class II antigens in normal and pathological human brain. Neuropathol Appl Neurobiol. 18:13.

13. Sedgwick, J.D., S. Schwender, R. Gregersen, R. Dorries, and V. ter Meulen. 1993. Resident macrophages (ramified microglia) of the adult brown norway rat central nervous system are constitutively major histocompability complex class II positive. J Exp Med 177:1145.

14. Hayes G.M., M.N., Woodroofe, and M.L. Cuzner. 1987. Microglia are the major cell types expressing MHC class II in human white matter. J Neurol Sci 80:25.

15. Boyle E.A., and P.L. McGeer. 1990. Cellular immune response in multiple sclerosis plaques. Am J Pathol 137:575.

16. Fontana A., W. Fierz, and H. Wekerle. 1984. Astrocytes present myelin basic protein to encephalitogenic T-cell lines. Nature 307:273.

17. Massa P.T., V. ter Meulen, and A. Fontana. 1987. Hyperinducibility of Ia antigen on astrocytes correlates with strain-specific susceptibility to experimental autoimmune encephalomyelitis. Proc Natl Acad Sci (USA) 84:4219.

18. Fierz W., B. Endler, K. Reske, H. Wekerle, and A. Fontana. 1985. Astrocytes as antigen-presenting cells. I Induction of Ia antigen expression on astrocytes by T cells via immune interferon and its effect on antigen presentation. J Immunol 134:3785.

19. Frei K., C. Siepl, P. Groscurth, S. Bodmer, C. Schwerdel, and A. Fontana. 1987. Antigen presentation and tumor cytotoxicity by interferon-gamma-treated microglial cells. Eur J Immunol 12:237.

20. Matsumoto Y., K. Ohmori, and M. Fujiwara. 1992. Immune regulation by brain cells in the central nervous system: microglia but not astrocytes present myelin basic protein to encephalitogenic T cells under in vivo- mimicking conditions. Immunol 76:209.

21. Williams K., A. Bar-Or, E. Ulvestad, A. Olivier, J.P. Antel, and V.W. Yong. 1992. Biology of adult human microglia in culture: comparisons with peripheral blood monocytes and astrocytes. J Neuropath Exp Neurol. 51:538.

22. Sedgwick J.P., R. MoBner, S. Schwender, and V. ter Meulen. 1991. Major histocompatibility complex-expressing non-haematopoietic astroglial cells prime only $CD8^+$ T lymphocytes/astroglial cells as perpetuator but not initiators of $CD4^+$ T cell responses in the central nervous system. J Exp Med 173:1235.

23. Williams K., E. Ulvestad, M. Blain, and J.P. Antel. 1993. Induction of primary T cell responses by human glial cells. J Neurosci Res 36:

24. Yong V.W., F.P. Yong, T.C.G. Ruijs, J.P. Antel, and S.U. Kim. 1991. Expression and modulation of HLA-DR on cultured human adult astrocytes. J. Neuropath Exp Neurol 50:16.

25. Yong V.W., and J.P. Antel. 1992. Culture of glial cells from human biopsies. In A. Richardson, and S. Fedoroff (eds.): Protocols for neural cell culture. St. Louis: Humana Press, 81-86.

26. Chomczynski P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 162:156.

27. Singer-sam J., M.O. Robinson, A.R. Bellve, M.I. Simon, and A.D. Riggs. Measurement by quantitative PCR of changes in HPRT, PGK-1, APRT, MTases and Zfy gene transcripts during mouse spermatogenesis. Nucleic Acid Res 18:1255.

28. Wang A.M., M.V. Doyle, and D.F. Mark. 1989. Quantitation of mRNA by the polymerase chain reaction. Proc Natl Acad Sci (USA). 86:9717.

29. Hirano T., K. Yasukawa, H. Harada, T. Taga, Y. Watanabe, T. Matsuda, H. Matsui, Y. Takahara, T. Taniguchi, and T. Kshimoto. 1986. Complementary DNA for a novel human interleukin (BNF-2) that induces B lymphocytes to produce immunoglobulin. Nature 324:73.

30. Arcari P., R. Martinelli, and F. Salvatore. 1984. The complete sequence of a full length cDNA for human liver glyceraldehyde-3-phosphate dehydrogenase: evidence for multiple mRNA species. Nucleic Acids Res 12:9179.

31. Gearing A.J.H., C.R. Bird, A. Bristow, S. Poole, and R. Thorpe. 1987. A simple sensitive bioassay for interleukin-1 which is unresponsive to 10^3 U/ml of interleukin-2. J Immunol Meth. 99:7.

32. Mosmann T.R., H. Cherwinski, M.W. Bond, M.A. Giedlin, R.L. Coffman. 1986. Two types of murine helper T cell clones. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol. 136:2348.

33. Aarden L.A., E.R. De Grot, O.L. Schaap, and P.M. Lansdorp. 1987. Production of hybridoma growth factor by human monocytes. Eur J Immunol. 17:1411.

34. Espevik T., and J. Nissen-Meyer. 1986. A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. J Immunol Meth. 95:99.

35. Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Meth. 65:55.

36. Lampson L. 1987. Molecular basis of the immune response to neural antigens. Trends Neurosci 10:211.

37. Rapoport S.I. 1976. Blood-brain barrier in physiology and medicine. New York:Raven Press.

38. Lassmann H., M.Schmied, K.Vass, and W.F. Hickey. 1993. Bone marrow derived elements and resident microglia in brain inflammation. Glia 7:19.

39. Sobel R.A., B.W. Blanchette, A.K. Bhan, and R.B. Colvin. 1984. The immunopathology of experimental allergic encephalomyelitis. II Endothelial cell Ia increases prior to inflammatory cell infiltrate. J Immunol. 132:2402.

40 Cserr H.F. and P.M. Knopf. 1992. Cervical lymphatics, the blood brain-barrier and the immunereactivity of the brain: a new view. Immunol Today 13:507.

41. Hickey W.F., J.P. Osborn, and W.M. Kirby. 1985. Expression of Ia molecules by astrocytes during acute experimental allergic encephalomyelitis in the lewis rat. Cell Immunol 91:528.

42. Matsumoto Y., N. Hara, R. Tanaka, and M. Fujiwara. 1986. Immunohistochemical analysis of the rat central nervous system during experimental allergic encephalomyelitis, with special reference to Ia- positive cells with dendritic morphology. J Immunol. 136:3668.

43. Sprent J., and M. Schaefer. 1989. Antigen presenting cells for unprimed T cell. Immunol Today. 10:17.

44. Altmann D.M., N. Hogg, J. Trowsdale, and D. Wilkinson. 1989. Cotransfection of ICAM-1 and HLA-DR reconstitutes human antigen-presenting cell function in mouse L cells. Nature 338:512.

45. Kanner S.B., N.K. Damle, J. Blake, A. Arruffo, and J.A. Ledbetter. 1992. CD2/LFA-3 ligation induces phospholipase C gamma tyrosine phosphorylation and regulates CD3 signalling. J Immunol 148:2023.

46. Linsley P.S., E.A. Clark, and J.A. Ledbetter. 1990. The T-cell antigen CD28 mediates adhesion with B cells by interacting with activation antigen B7/BB-1. Proc Natl Acad Sci (USA) 87:5031.

47. Houssia F.A., P. Coule, and J. Van Snick. 1989. Distinct roles of IL-1 and IL-6 in human T cell activation. J Immunol. 143:2514.

48. Vink A., C. Uyttenhove, P. Wauters, and J. Van Snick. 1990. Accessory factors involved in murine T cell activation. Distinct roles of interleukin 6, interleukin 1 and tumor necrosis factor. Eur. J Immunol. 20:1.

49. Hetier E., J. Ayala, P. Denefe, A Bosseau, P Rouget, M. Mallat, and A. Prochiantz. 1988. Brain macrophages synthesize interleukin-1 and interleukin-1 mRNA in vitro. J Neurosci Res. 21:391.

50. Giulian D., T.J. Baker, L.C.N. Shih, and L.B. Lachman. 1986. Interleukin 1 of the central nervous system is produced by ameboid microglia. J Exp Med 164:594.

51. Woodroofe M.N., G.S. Sarna, M. Wadhwa, G.M. Hayes, A.J. Loughlin, A. Tinker, and M.L. Cuzner. 1991. Detection of interleukin-1 and interleukin-6 in adult rat brain, following mechanical injury, by in vivo microdialysis: evidence of a role for microglia in cytokine production. J Neuroimmunol. 33:227.

52. Sebire G., D. Emilie, C. Wallon, C. Hery, O. Devergne, J.F. Delfraissy, P. Galanaud, and M. Tardieu. 1993. In vitro production of IL-6, IL-1ß, and tumor necrosis factor-α by human embryonic microglial and neural cells. J Immunol 150:1517.

53. Lee S.C., W. Liu, D.W. Dickson, C.F. Brosnan, and J.W. Berman. 1993. Cytokine production by human fetal microglia and astrocytes. Differential induction by lipopolysaccharide and IL-1ß . J Immunol. 150:2659.

54. Aloisi F., A. Care, G. Borsellino, P. Gallo, S. Rosa, A. Bassani, A. Cabibbo, U. Testa, G. Levi, and C. Peschle. 1992. Production of hemolymphopoietic cytokines (IL-6, IL-8, colony-stimulating factors) by normal human astrocytes in response to IL-1ß and tumor necrosis factor-α. J Immunol. 149:2358.

55. Fontana A., F. Kristensen, R. Dubs, D. Gemsa, and E. Weber. 1982. Production of prostaglandin E and interleukin-1 like factor by cultured astrocytes and C_6 glioma cells. J Immunol. 129:2413.

56. Fontana A., K.P.W.J. McAdam, F. Kristensen, E. Weber. 1983. Biological and biochemical characterization of an interleukin 1-like factor from rat C6 glioma cell. Eur J Immunol. 13:685.

57. Fontana A., and P.J. Grob. 1984. Astrocyte derived interleukin-1-like factors. Lymphokine Res. 3:11.

58. Lieberman A.P., P.M. Pitha, H.S. Shin, and M.L. Shin. 1989. Production of tumor necrosis factor and other cytokines by astrocytes stimulated with lipopolysaccharide or a neurotropic virus. Proc Natl Acad Sci (USA) 86:6348.

59. Sawada M., N. Kondo, A. Suzumura, and T. Marunouchi. 1989. Production of tumor necrosis factor-alpha by microglia and astrocytes in culture. Brain Res. 491:394.

60. Hofman F.M., R.G. von Hanwehr, C.A. Dinarello, S.B. Mizel, D. Hinton, and J.E. Merril. 1986. Immunoregulatory molecules and IL-2 receptors identified in multiple sclerosis brain. J Immunol. 135:3239.

61. Tsukada N., K. Miyagi, M. Matsuda, N. Yanagisawa, and K, Yone. 1991. Tumor necrosis factor and interleukin-1 in the CSF and sera of patients with multiple sclerosis. J Neurol Sci. 102:230.

62. Dinarello C.A. 1991 Interleukin-1 and interleukin-1 antagonism. Blood 77:1627.

63. DeFreitas E.C., R.W. Chesnut, H.M. Grey, and J.M. Chiller. 1983. Macrophage-dependent activation of antigen-specific T cells require antigen and a soluble monokine. J Immunol. 131:23.

64. Kurt-Jones E.A., D.I. Beller, S.B. Mizel, and E.R. Unanue. 1985. Identification of a membrane-associated interleukin 1 in macrophages. Proc Natl Acad Sci (USA) 82:1204.

65. Scala G., and J.J. Oppenheim. 1983 Antigen presentation by human monocytes: evidence for stimulant processing and requirement for interleukin 1. J Immunol 131:1160.

66. Chu E.T., M. Lareau, L.J. Rosenwasser, C.A. Dinarello, and R.S. Geha. 1985. Antigen presentation by EBV-B cells to resting and activated T cells: role of interleukin 1. J Immunol. 134:1676.

67. Tartakovsky B., A. Finnegan, K. Muegge, D.T. Brody, E.J. Kovacs, M.R. Smith, J.A. Berzofsky, H.A. Young, and S.K. Durum. 1988. IL-1 is an autocrine growth factor for T cell clones. J Immunol 141:3863.

68. Weaver C.T., C.M. Hawryolxicz, and E.R. Unanue. 1988. T helper cell subsets require the expression of distinct costimulatory signals by antigen-presenting cells. Proc Natl Acad Sci (USA) 85:8181.

69. Zubiaga A.M., E. Munoz, and B.T. Hube. 1991. Production of IL-1α by activated Th type 2 cells. Its role as an autocrine growth factor. J Immunol. 146:3849.

70. Enk A.H., and S.I. Katz. 1992. Early molecular events in the induction phase of contact sensitivity. Proc Natl Acad Sci (USA) 89:1398.

71. Koide S., and R.M. Steinman. 1989. Induction of interleukin Iα mRNA during the antigen-dependent interaction of sensitized T lymphoblasts with macrophages. J Exp Med. 168:409.

72. Holsti M.A., and D. Raulet. 1989. IL-6 and IL-1 synergize to stimulate IL-2 production and proliferation of peripheral T cells. J Immunol. 143:2514.

73. Miller JFAP, G. Morahan, J. Allison, and M. Hoffman. A transgenic approach to the study of peripheral tolerance. Immunol Rev 122:103.

74. Harding F.A., J.G. McArthur, J.A. Cross, D.H. Raulet, J.P. Allison. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T cell clones. Nature 19:356:607.

TABLE I

IL-1 reverses the inability of human fetal astrocytes to present antigen in the MLR.

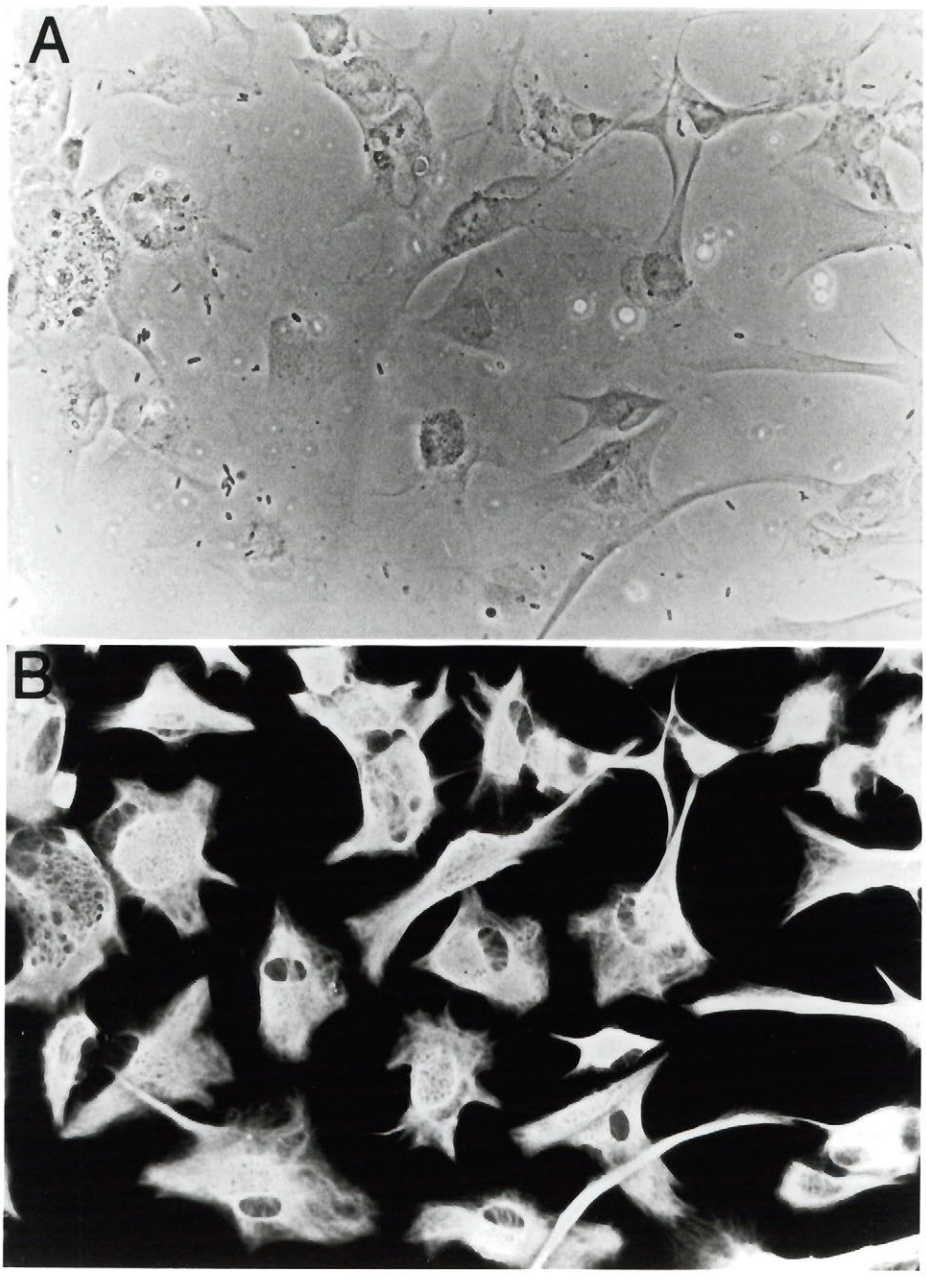
	Ast + CD4$^+$T			Ast(IFN) + CD4$^+$T		
	No IL-1	10 U/ml	50 U/ml	No IL-1	10 U/ml	50 U/ml
1a	1,016 ±133	-- --	2,388 ±1,510	2341 ±920	-- --	10,555 ±2,880
b	302 ±53	-- --	7,222 ±112	1,419 ±495	-- --	14,296 ±2,573
2a	124 ±12	-- --	-- --	892 ±524	1,110 ±201	5,268 ±211
b	-- --	-- --	-- --	524 ±201	1,795 ±841	15,755 1,118
3a	206 ±46	29,570 ±3,289	30,337 ±5,293	892 ±536	46,332 ±9,861	52,612 ±8,116
b	710 ±33	12,180 ±1,026	15,110 ±1,555	860 ±536	23,533 ±5,264	26,455 ±1,557
4a	848 ±405	11,688 ±5,884	31,961 ±8,001	524 ±232	35,398 ±1,805	38,934 ±1,412
b	192 ±13	3,605 ±602	4,018 ±392	501 ±198	6,210 ±839	15,048 ±2,285

Fresh CD4$^+$ T cells ($1X10^5$) were cocultured with astrocytes ($2X10^4$). In some experiments rh IL-1α was added concurrently with CD4$^+$ T cells. Astrocytes were either under basal culture conditions (Ast) or stimulated with IFN (Ast/IFN). Cultures were harvested on day 5 after a 5 hr pulse with [^{3}H] TdR. Data represent cpm ± sem of triplicate cultures. cpm of astrocytes or CD4$^+$ T cells alone was <900. Data represent N=4 experiments with 2 CD4$^+$ T cell donors (a,b) per assay.

FIGURE LEGENDS

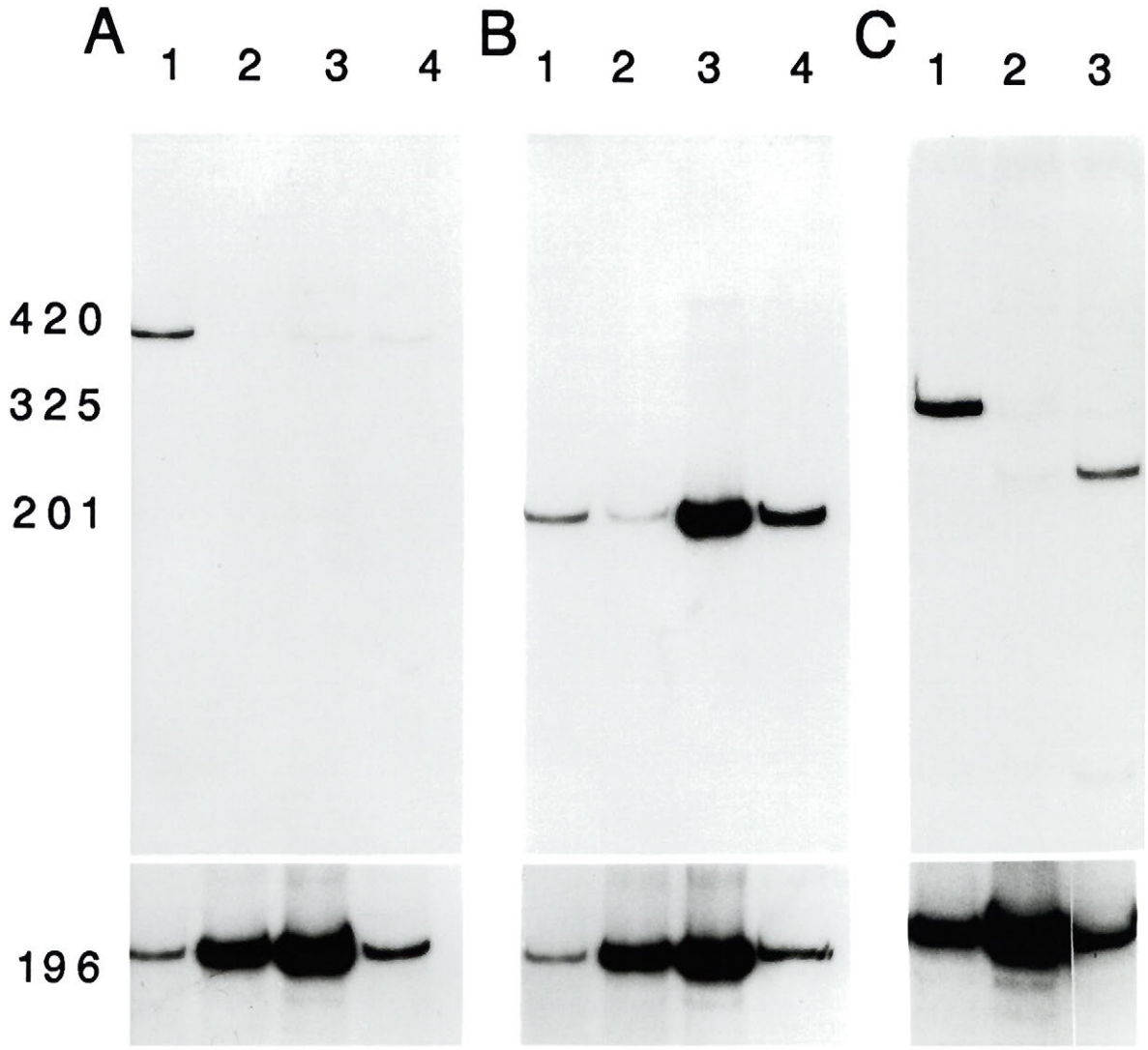
Figure 1. Adult human derived microglia in vitro (enriched cultures >95% purity). Leu-M5 immunoreactivity was demonstrated by fixing cells with ice-cold acetone for 5 min, incubating with primary Ab overnight, followed by biotinylated rabbit anti-mouse immunoglobulin (1:300) for 30 min, and avidin-biotin-peroxidase complex (ABComplex/HRP) for 30 min. The colored reaction product was developed using 3-amino-9-ethyl-carbazole-containing buffer (300X mag).

Figure 2. Fetal human derived astrocytes in vitro. A: Phase contrast photomicrograph of enriched (>95% purity) astrocyte culture (300X mag). B: GFAP immunoreactivity of same field (300X mag).


A
B

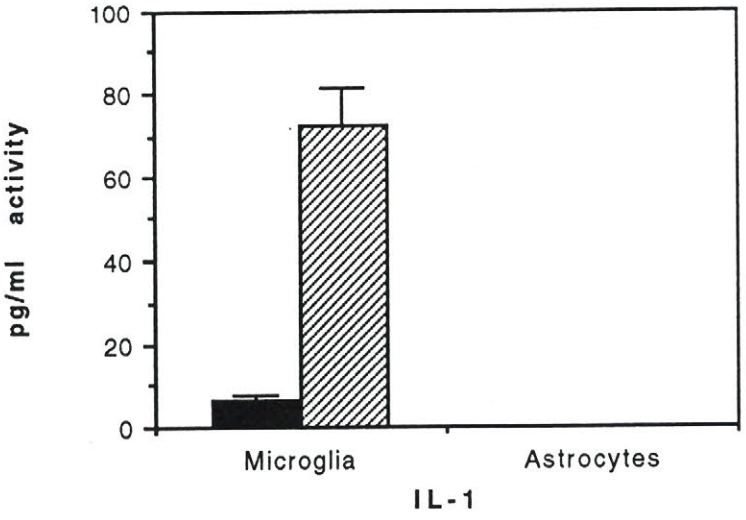
93-1396

Figure 3. cDNA corresponding to IL-1α, IL-6, and TNFα mRNA expression by enriched microglia and astrocyte in vitro. Cytokine mRNA was amplified by reverse transcriptase-coupled PCR (as described in methods). The autoradiogram depicts signal for A: IL-1α (expected product 420 bp) by microglia (lane 1) with no detectable expression by astrocytes under basal culture conditions (lane 2) or following IFN gamma stimulation (lane 3) or in a snap frozen brain specimen (lane 4). B: IL-6 (expected product 201 bp) by microglia, astrocytes under basal and activation conditions, and in frozen brain specimen (lane 1 through 4 respectively). C: TNFα (expected product 325 bp) by microglia (lane 1) but not by IFN activated astrocytes (lane 2) or frozen brain specimen (lane 3). G3DPH (expected product 196 bp) served as a control.

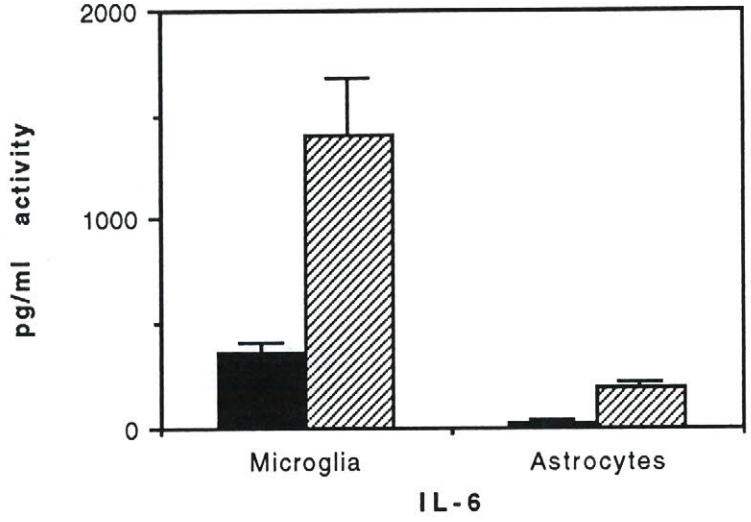

A
1 2 3 4
B
1 2 3 4
C
1 2 3
420
325
201
196

92-1830

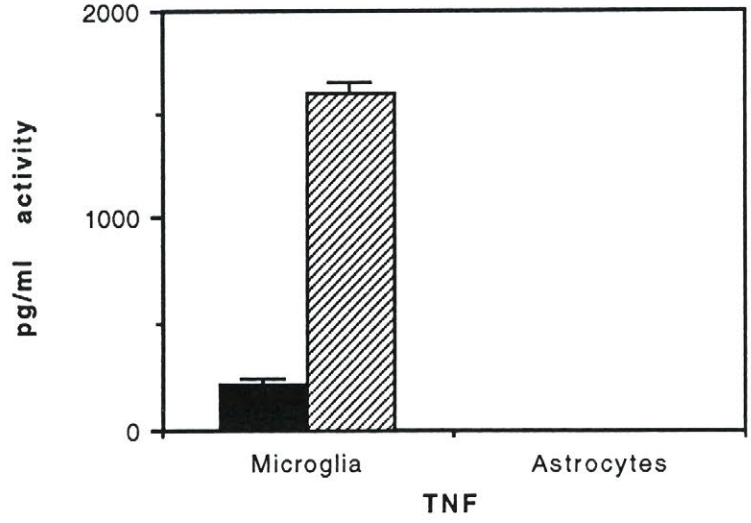
Figure 4. Cytokine production by adult human derived microglia and fetal human derived astrocytes in vitro. Data represent cytokine production by microglia and astrocytes seeded at a concentration of 4 X 10^4 cells per well. Cytokine production was 1 ml of conditioned supernatant of glial cells under basal culture conditions (solid bar) and following IFN gamma stimulation (hatched bar). Data represent the mean $\pm$ sem pg/ml protein of triplicate wells (n=2 experiments).


100
80
60
40
20
0
pg/ml activity
Microglia
Astrocytes

IL-1


2000
1000
0
pg/ml activity
Microglia
Astrocytes

IL-6


2000
1000
0
pg/ml activity
Microglia
Astrocytes

TNF

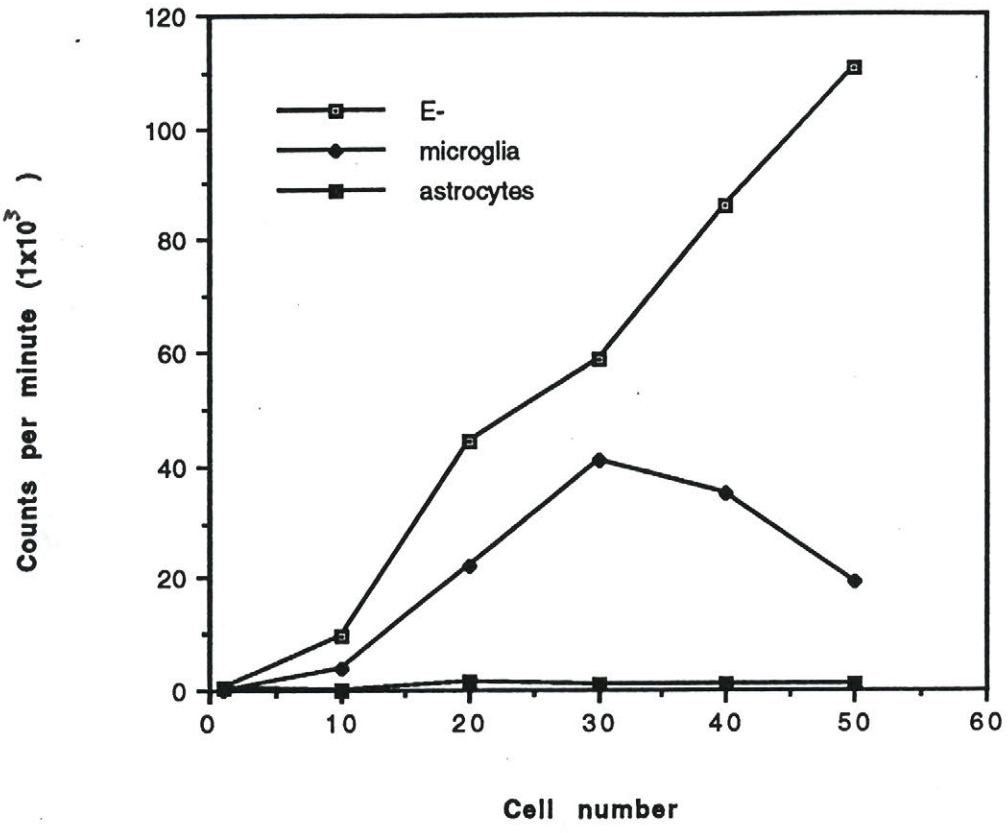


Figure 5. Titration curve demonstrating stimulation of fresh allogeneic $CD4^+$ T cells by microglia and non-rosetted peripheral blood derived cells (E-), but not astrocytes. $CD4^+$ T cells ($1X10^5$) were cocultured with irradiated astrocytes, microglia, and E^- cells seeded at densities ranging from 10^2 to $5X10^4$ cell per well. Cultures were harvested on day 5 after a 5 hr pulse with [^{3}H] TdR. Data points represent cpm $\pm$ sem of triplicate cultures. cpm of astrocytes, microglia, E^-, and T cells alone were <900. (n=2 experiments).

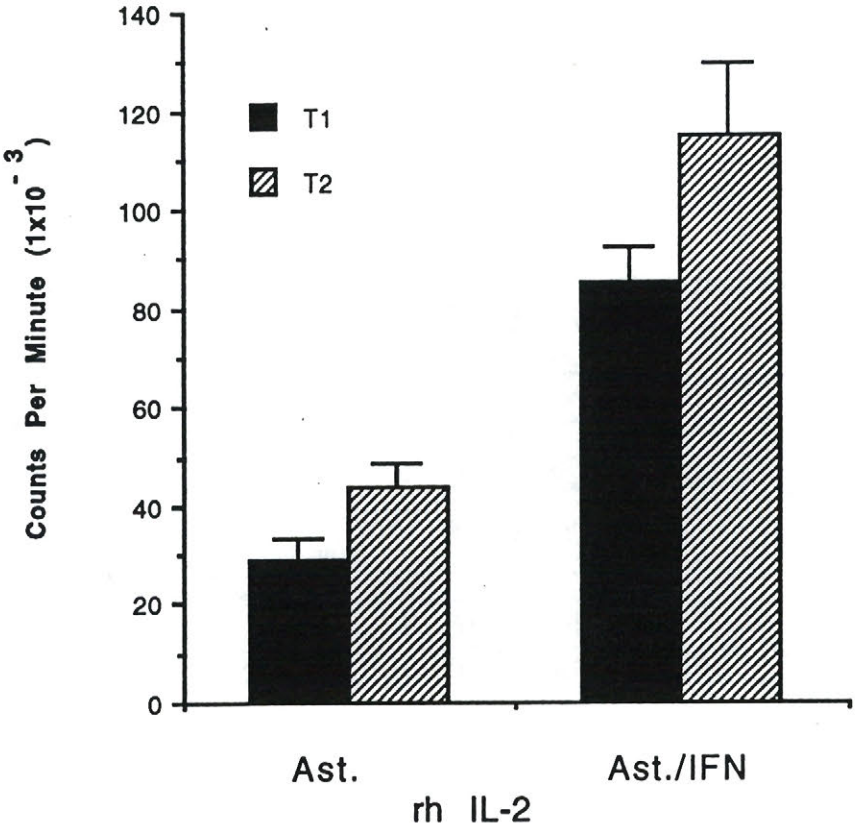


Figure 6. Astrocytes stimulate $CD4^+$ T cells to proliferate in the presence of exogenous rIL-2. Fresh $CD4^+$ T cells ($1X10^5$) from 2 donors per experiment (donor 1 = solid bar, donor 2 = hatched bar) were cocultured with $2X10^4$ astrocytes. Some astrocyte cultures were incubated with IFN (100 U/ml) for 48 hr prior to the addition of T cells. Cultures were harvested on day 5 after a 5 hr pulse with [^{3}H] TdR. Bars depict cpm $\pm$ sem for triplicate cultures. cpm of astrocyte and $CD4^+$ T cells alone <900. Data presented here are representative of n=4 experiments.

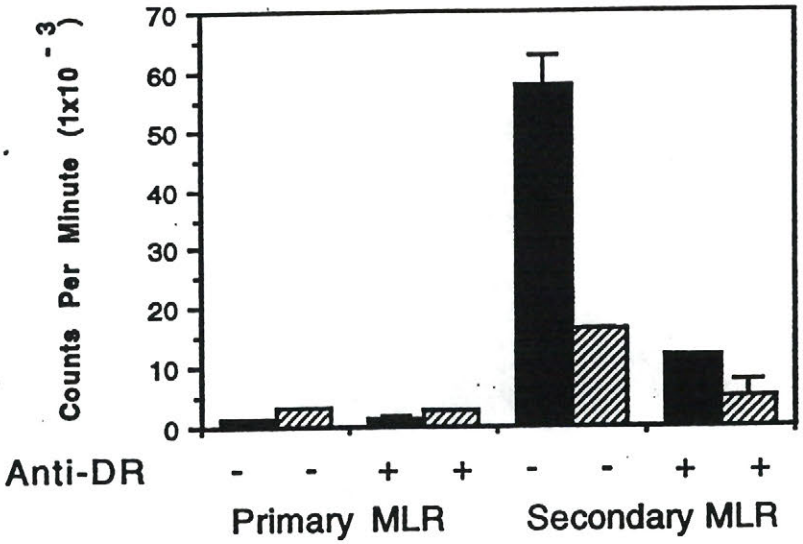


Figure 7. Astrocytes can support the continued proliferation of previously activated $CD4^+$ T cells, but can not present antigen in the MLR. Fresh $CD4^+$ T cells (1° MLR) and $CD4^+$ T cells from the same donor that had been previously cocultured with sister astrocyte in the presence of IL-2 (50 U/ml) for 5 days, recovered, and cocultured with astrocytes ($2X10^4$)(2° MLR). Some astrocyte cultures were preincubated with an anti-HLA-DR mAb (L243 20μg/ml, 30 min on ice) prior to the addition of $CD4^+$ T cells. Cultures were harvested on day 5 after a 5 hr pulse with $[^3H]$ TdR. Bars depict cpm $\pm$ sem of triplicate cultures using 2 different sources of $CD4^+$ T cells per experiment (donor 1 = solid bar; donor 2 = hatched bar). cpm of astrocytes or $CD4^+$ T cells alone <900. Results presented here are representative of n=3 experiments.

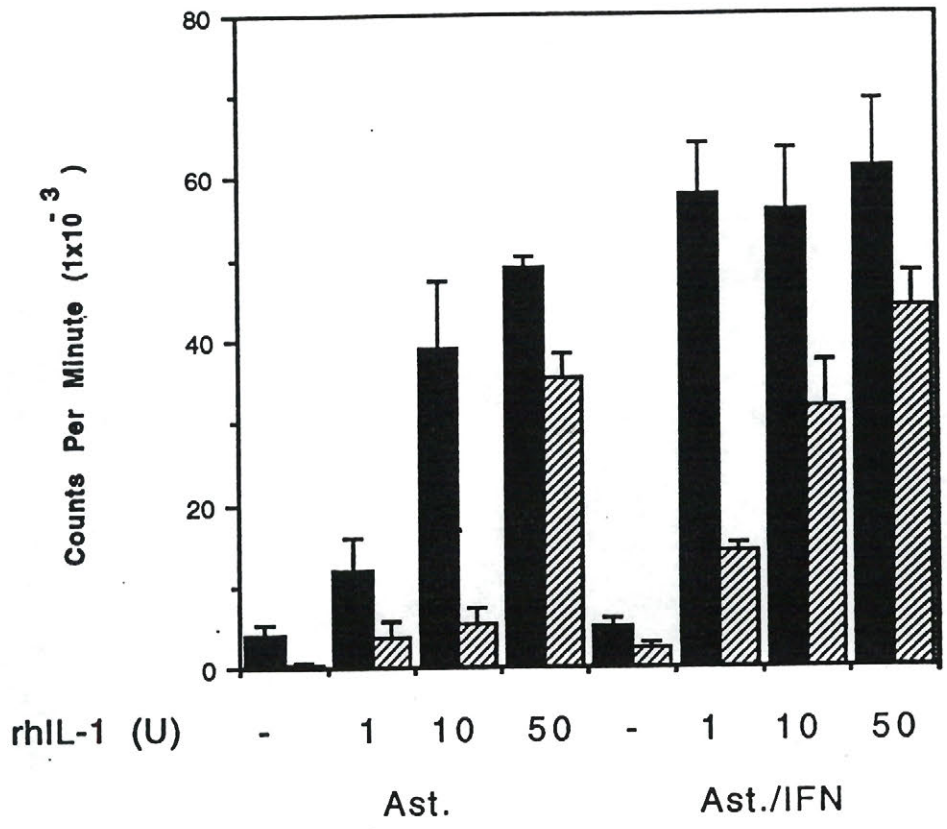


Figure 8. Astrocytes can present Ag in the allogeneic MLR with the addition of rIL-1. Fresh $CD4^+$ T cells ($1X10^5$) from 2 different donors (donor 1 = solid bar; donor 2 = hatched bar) cocultured with astrocytes ($2X10^4$) under basal (Ast) and IFN activation conditions (Ast/IFN). rh IL-1 (1-50 U/ml) was added to cocultures at the beginning of the assay and cultures were harvested on day 5 after a 5 hr pulse with [^{3}H] TdR. Bars represent cpm $\pm$ sem for triplicate cultures. cpm of astrocytes or $CD4^+$ T cells alone were <900.

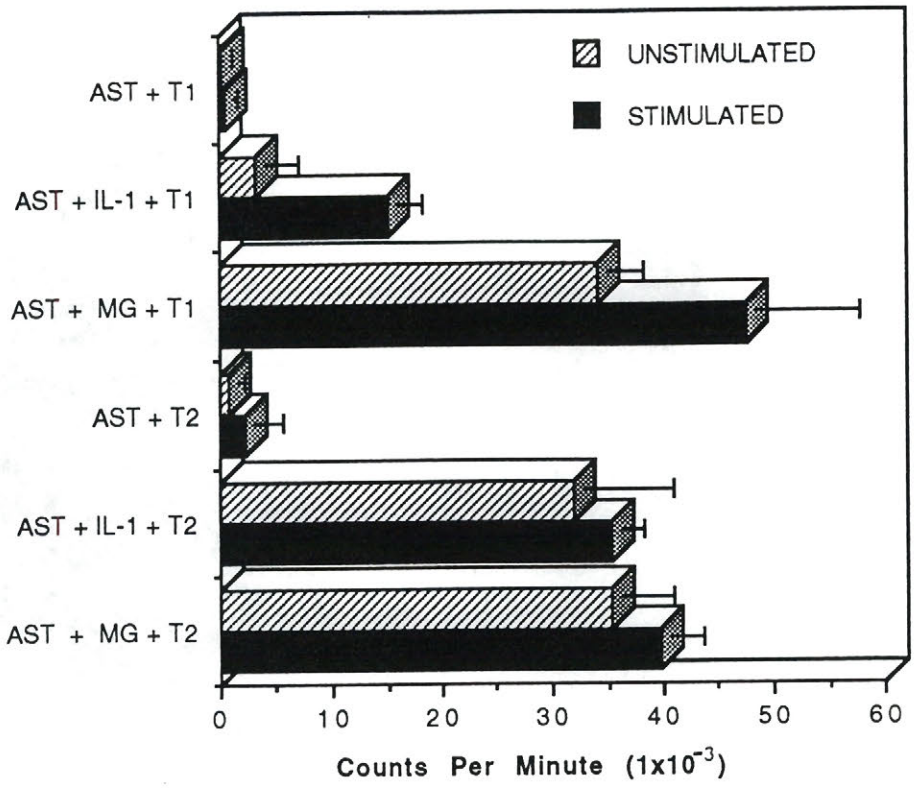


Figure 9. Astrocytes can present Ag in the MLR with the cooperation of rh IL-1α or microglia. Fresh CD4$^+$ T cells (1X10^5) from 2 donors (donor 1=T1, donor 2=T2) were cocultured with astrocytes (2X10^4) under basal culture conditions (hatched bars) and following IFN stimulation (solid bars). In some experiments IL-1 (10 U/ml) or irradiated microglia (10^3) (MG) were added to astrocyte: CD4$^+$ T cell cocultures. Cultures were harvested on day 5 after a 5 hr pulse with [^{3}H] TdR. cpm of astrocytes, astrocytes plus microglia, or CD4$^+$ T cell with rh IL-1 were $<$900. Results presented here are representative of n=4 experiments.

Chapter 6

B7/BB-1 Antigen Expression on Adult Human Microglia studied in vitro and in situ

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Preface

This chapter explored the functional significance of B7/BB-1 antigens expressed on microglia in vitro using a mAb and a fusion protein to bind to the B7/BB-1 molecule to inhibit microglial/T cell interactions. Additionally, studies were undertaken investigating the expression of B7/BB-1 on normal and MS brain sections. Major points demonstrated in this chapter include:

1. Confirmation of the expression of B7/BB-1 antigen on microglia with upregulation of expression following IFN gamma treatment or co-culture of $CD4^+$ T cells.

2. The demonstration of B7/BB-1 on microglia in active MS lesions but not on adjacent, non lesion areas. B7/BB-1 was also demonstrated on perivascular microglia and infiltrating cells in perivascular cuffs.

3. The demonstration that pre-incubation of microglia with an anti-B7/BB-1 mAb or

the CTLA-4 Ig fusion protein partially inhibited the ability of microglia in vitro to present antigen in a primary MLR; to present recall antigens; and to support the continued proliferation of previously activated $CD4^+$ T cells in a 2° MLR. Pre-incubation of astrocytes with the same anti BB-1 mAb did not result in the inhibition of the B7/BB-1 negative astrocytes to support the continued proliferation of previously activated T cells.

ABSTRACT

In this study, we have examined the expression and function of B7/BB-1 antigen on individual human glial cells in vitro and in situ, by utilizing surgically resected adult human central nervous system (CNS) tissue, abortus derived fetal human CNS tissue, and pathology material from cases of multiple sclerosis (MS). Immunofluorescence analysis using enriched adult human derived cultures of microglia and oligodendrocytes, and mixed microglia/astrocytes cultures, demonstrated B7/BB-1 on microglia following stimulation with interferon (IFN) gamma. Oligodendrocytes and astrocytes, as well as human fetal astrocytes were B7/BB-1 negative under all culture conditions. Flow cytometry studies showed a low basal level of B7/BB-1 expression on microglia that was upregulated following IFN gamma incubation. Co-culture of purified fresh $CD4^+$ T cells with microglia for 24 hours resulted in clustering of T cells around microglia and microglial B7/BB-1 expression. Pre-incubation of microglia with an anti BB-1 mAb prior to microglia: $CD4^+$ T cell co-cultures resulted in partial inhibition of the ability of microglia both to present recall antigen to autologous T cells and to a lesser extent inhibited the ability of microglia to present antigen to allogeneic $CD4^+$ T cells in a primary mixed lymphocyte reaction (MLR). The CTLA-4 Ig fusion protein inhibited the ability of microglia to present antigen in both antigen presentation assays to an even greater extent than did the anti BB-1 mAb. The BB-1 antibody also inhibited the ability of microglia, but not of the B7/BB-1 negative astrocytes,

to stimulate previously activated T cells in a secondary MLR. In sections of multiple sclerosis brain, B7/BB-1 expression was observed on activated microglia in select parenchymal lesions, and on perivascular cells and infiltrating monocytes. No B7/BB-1 immmunoreactivity was found in normal appearing white matter or in non-inflammatory brain specimens. Our results indicate that the B7/BB-1 molecule plays a functional role in the capacity of microglia to serve as CNS antigen presenting cells and thus, may promote immune reactivity within the CNS during the course of autoimmune disease.

INTRODUCTION

The B7/BB-1 Ag is constitutively expressed on dendritic cells and on activated B cells and macrophages (1-3). B7/BB-1 expression is upregulated on B cells following stimulation with anti-IgG (1) and on monocytes following stimulation with interferon gamma (IFN) (4). B7 binds to CD28 (5) expressed on the majority of $CD4^+$ T lymphocytes (6) and to the CD28 homologue CTLA-4 expressed on activated T cells (7). Ligation of CD28 by B7 has been demonstrated to augment T cell activation by a T cell receptor independent mechanism, resulting in the induction of IL-2 transcription and mRNA stabilization (8,9)

We have previously reported that adult human derived microglia can both present recall antigen to immediately ex-vivo $CD4^+$ T cells with resultant T cell proliferation and present antigen in a primary mixed lymphocyte reaction system (MLR) (10,11). Human fetal astrocytes were unable to present antigen in the primary MLR, but could support the continued proliferation of previously activated $CD4^+$ T cells (Williams et al., Submitted for publication). Although human microglia and astrocytes both express an array of immune adhesion molecules including ICAM-1, and LFA-3, we previously found that microglia but not astrocytes, express B7/BB-1 in vitro following IFN gamma incubation (11). In this report we have investigated the expression and functional significance of B7/BB-1 by microglia in vitro and in situ. In vitro, a negligible-to-low level of B7/BB-1 antigen expressed on microglia could be augmented both with IFN incubation and following co-culture of microglia with $CD4^+$ T cells. Pre-incubation of microglia with the anti-BB-1 mAb inhibited the ability of microglia to present recall antigen, to present antigen in a MLR, and the ability of microglia to support the proliferation of previously activated T cells. The CTLA-4 Ig fusion protein was able to inhibit both the recall and primary MLR response by microglia to an even greater extent than the anti-BB-1 mAb. In situ, expression of B7/BB-1 antigens was found on microglia in active MS lesions but not in parenchymal material outside of and adjacent to active lesions. These data are discussed in terms of microglial cells functioning as CNS antigen presenting cells (APC) and the role of

B7 in augmenting CNS immune activity.

Material and Methods

Tissue

CNS tissue was removed from patients undergoing surgical resections for treatment of intractable epilepsy (N=6) (age range 22-36 years) (pathology ranged from mild to severe gliosis without evidence of inflammation or malignancy). Tissue was removed by Cavitron ultrasonic aspiration resulting in 2-3 mm^3 pieces, some of which were blocked in OCT cryomount (Tissu-Tek, Elkhart, IN) and frozen in isopentane previously cooled in liquid nitrogen, and then sectioned (4-6 μm) on a cryostat. Brain specimens from 2 patients with multiple sclerosis were obtained at autopsy and lesion and non-lesion areas were similarly frozen in liquid nitrogen and sectioned. Fetal human CNS tissue served as the source of astrocytes and were prepared from 8-12 week old specimens (n=3) following Medical Research Council of Canada approved guidlines.

Glial cell cultures

Isolated cells from adult human tissue were obtained by a previously described protocol (12). In brief, aspirated tissue was washed with PBS and dissociated using 0.25% trypsin in the presence of 20 μg/ml DNase (GIBCO, Burlington, Ont) for 1 hr at 37° C. The resulting cell suspension was then centrifuged in 30% Percoll at 4° C. Glial cells were recovered, washed in PBS and seeded in Eagles minimum essential medium supplemented with 5% FCS, Gentamicin 20 μg/ml, glucose 1μg/ml and 2 mM glutamine (GIBCO) into 25 cm^2 culture flasks (Nunc, Burlington, Ontario) or directly onto 9mm Aclar fluorocarbon plastic coverslips (S. Kim, Vancouver, Canada) previously coated with 10 μg/ml poly-l-lysine (Sigma, St Louis, MO). Cells in flasks were left undisturbed overnight at 37° C in a humidified incubator with 5% CO_2. The following day, culture flasks were lightly shaken and floating cells consisting of > 80% oligodendrocytes were removed and seeded onto coverslips as described above. The

remaining cells consisting of mixed microglia and astrocytes were either retrypsinized and seeded onto coverslips as mixed microglia-astrocyte cultures or they were allowed to differentiate morphologically for 1 week. After 1 week, enriched microglia cultures were obtained by subjecting mixed microglia-astrocyte cultures to 5 hr 150 rpm shaking on an orbital shaker. The less adherent astrocytes floated off and remaining microglia (>95% Leu-M5 $^+$ cells) were either seeded onto coverslips for immunofluorescence studies, trypsinized from flasks and stained for flow cytometry, or seeded into 96 well microtiter plates for functional studies.

Fetal astrocyte culture were prepared by stripping CNS materials of meninges and blood vessels and dissociating the tissue first mechanically and then using 0.25% trypsin in the presence of DNase (50 μg/ml) at 37° C for 45 min. The tissues were passed through a 130 μm nylon mesh and then washed 2X in PBS. Dissociated cells were then plated onto poly-l-lysine coated culture dishes in culture medium described above. Cultures were split every 2 weeks when confluent and experiments conducted after the second or third passage when neurons and microglia were no longer apparent. For APC assays, astrocytes were lightly trypsinized 0.05% trypsin for 15 min a 37°C, irradated (2,500 rads), and seeded into 96 well plates at a density of $2X10^4$ cell per well.

CD4$^+$ Lymphocyte Preparations

Mononuclear cells (MNC) and CD4$^+$ T cell populations were isolated and prepared from, either individuals whose resected CNS tissue was used to establish microglia cultures or from healthy normal individuals as previously described (10,11). Briefly, MNC's were isolated by Ficoll-Hypaque density centrifugation from heparinized peripheral blood samples. Total T cells were isolated by rosetting MNCs with S-(2-aminoethyl isothiouronium bromide) (AET)- treated sheep red blood cells (13). CD4$^+$ T cells were then prepared by complement mediated lysis of CD8$^+$ cells using OKT8 mAb (hybridoma obtained from ATCC, Rockville, MD) followed by the addition of rabbit complement (Cedarlain Laboratories, Hornby, Ontario). T cells were cultured in medium consisting of RPMI supplemented with 5% human AB serum

(Pel-Frez, Deer Born, WI), 2.5 μg/ml penicillin, and 2.5 μg/ml streptomycin. The mean purity of $CD4^+$ T cells, assessed by FACscan analysis of T cells immunostained with phycoerythrein-conjugated anti-CD4 mAb (Leu3a) (Becton-Dickinson, San Jose, CA) and fluorescein-labelled anti-CD8 mAb (Leu2), was >96%.

Antigen presentation assays

Presentation of recall antigens

$1x10^5$ autologous peripheral blood derived $CD4^+$ T cells were co-cultured in 96 well microtitre plates with $1x10^4$ irradiated microglia (2,500 rad) in 200 μl of culture medium. 10 μl of a Tetanus toxoid antigen stock solution (1:100 v/v dilution of a 50 protein unit per ml stock) was added to individual wells at the beginning of the assay.

Primary mixed lymphocyte cultures

$1x10^5$ allogeneic $CD4^+$ T cells (from 2 different donors per experiment) were co-incubated with irradiated microglia which had been previously seeded in 96 well microtitre plates at a concentration of $2.5x10^4$ cells per well. In some experiments, non-rosetting mononuclear cells (E^- cells) consisting of monocytes and B lymphocytes, were irradiated (2,500 rads) and used as stimulator cells ($1x10^5$ cells per well) in the MLR. Medium for all glial: T cell antigen presentation assays was RPMI 1640 supplemented with 5% human AB serum (Pel Frez). Recall antigen assays and the MLR assay were carried out for 7 and 5 days respectively. At the end of the assay proliferation was measured following a 5 hour pulse with ^3H-thymidine uptake (1μCi per well) (ICN Flow Laboratories, Mississaga, Ontario). Cells were harvested and radioactive counts determined using a beta liquid scintillation counter (LKB, Fisher, Montreal, PQ). Results are expressed as mean counts per minute (CPM) of triplicated culture wells.

Activated T cell cultures

For these studies, purified $CD4^+$ T cells ($1x10^7$) were co-incubated with confluent allogeneic astrocyte or microglial cultures in 25 cm^2 Nunc tissue culture

flasks in the presence of exogenously added rh IL-2 (50 U/ml, Genzyme, Boston, MA) for 5 days. After 5 day in vitro, T cells were recovered, washed, and cultured with corresponding sister microglia or astrocytes in a MLR system as described above.

APC inhibition assays were performed by preincubating stimulator cells with either anti-BB-1 mAb (IgM, 10-100 μg/ml) (a gift from Dr. J Ledbetter, Bristol Meyers Squibb, Seattle, WA) the CTLA-4 Ig fusion protein CTLA-4 (2-10 μg/ml) (a gift from Dr. J Ledbetter), or the HLA-DR blocking mAb L243 (IgG2a, 20μg/ml) (a gift from Dr. Rafik Sekaly, CRIM, Montreal) at 4° C for 30 min. The percent of inhibition was calculated relative to the basal response of unblocked control cultures.

Immunofluorescence analysis

Cells on coverslips were analyzed by an indirect immunofluorescence labelling technique as previously described (10). Purity of individual glial cell populations was determined using either mouse anti-CD11c (microglia) (1:10, Becton-Dickinson), rabbit anti-GFAP (astrocytes) (1:100, Dako, West Chester, PA), or mouse anti-GalC (oligodendrocytes) (H8H9, 1:10, a gift from Dr S Kim, Vancouver, BC) followed by rhodamine conjugated goat anti-mouse antibodies (1:150, Cappel, Lexington, MA) or flourescein conjugated goat anti-rabbit antibodies (1:150, Capell). To immunostain enriched microglia and oligodendrocyte cultures for B7/BB-1, viable cells on coverslips were first incubated with the anti-B7/BB-1 mAb (1:100, Becton-Dickinson) for 1 hour at 4°C followed by rhodamine-conjugated goat anti-mouse ab for 45 min at 4°C, and then fixed using acid alcohol (5% glacial acetic acid: 95% absolute ethanol, v/v) at -20 °C for 15 min. Mixed microglia-astrocyte cultures under basal culture conditions and following activation with human rIFN gamma (100 U/ml, Genzyme) were double stained using a B7/GFAP protocol where viable cells on coverslips were first incubated with anti-B7/BB-1 (1:100, Becton-Dickinson) for 1 hr 4° C followed by rhodamine-conjugated goat anti-mouse ab for 45 min at 4° C, and then fixed and stained for GFAP as described above. Enriched microglial cultures were also analyzed for basal and IFN gamma (100 U/ml) induced levels of B7/BB-1 expression compared with basal

and induced HLA-DR (anti HLA-DR- FITC, Becton-Dickinson, 10 μg/ml)) by flow cytometry. For flow cytometry staining, microglia were trypsinized from culture flasks, washed 2x with PBS with 1% bovine serum albumin (BSA), incubated with primary mAb for 45 min on ice, washed 2x, and incubated with FITC conjugated goat anti-mouse IgG for 45 min on ice, washed and then fixed with a 1% solution of paraformaldehyde in PBS. Controls consisted of unstained cells, secondary antibody only, and irrelevant isotype controls.

Immunohistochemistry

Sections were fixed in cold acetone for 5 min. Following fixation, sections were incubated with 10% rabbit serum diluted in PBS, ph 7.2 for 30 min at room temperature followed by overnight incubation at 4° C in a humid chamber with mAbs diluted in 10% rabbit serum. Biotinylated rabbit anti-mouse immunoglobulin diluted 1:300 were applied for 30 min. Avidin-biotin-peroxidase complex (ABC complex/HRP, Dakopatts) was prepared according to manufacturers recommendation and allowed to react with sections for 30 min. Thereafter sections were treated with a 3-amino-9-ethyl-carbazole containing buffer and the colored reaction products monitored. Sections were lightly counterstained with haematoxylin, mounted in glycergel (Dakopatts) and examined by light microscopy. In addition to staining with B7/BB-1 mAb, alternate serial sections were stained with Leu-M3 mAb (CD14, Becton-Dickinson) and subjected to the non-specific esterase (NSE) reaction as previously described (10) to differentiate between infiltrating monocytes (Leu-M3$^+$, NSE$^+$) and microglia (Leu-M3$^-$, NSE$^-$) (14,15).

RESULTS

Expression and function of B7/BB-1 on microglia in vitro.

Enriched cultures of adult human derived microglia (>95% Leu-M5+) (Figure 1), oligodendrocytes (>80% GalC$^+$), and mixed microglia/astrocyte culture were established as previously reported (10-12). Microglia in either enriched cultures or in mixed astrocyte/microglial cultures were B7/BB-1 positive following IFN gamma incubation as assessed using fluorescence microscopy. Oligodendrocytes and astrocytes were B7/BB-1 negative under all culture conditions. Results from flow cytometry studies demonstrated a low basal level of B7/BB-1 by microglia with variability of expression between individual brain specimens (N=3) (Figure 2). HLA-DR was more readily detected on the microglia under basal culture conditions than was B7/BB-1 using flow cytometry (Figure 2); B7/BB-1 and HLA-DR expression both were increased on microglia following stimulation with IFN gamma (Figure 2). When $1x10^5$ CD4$^+$ T cells were co-incubated with $1x10^3$ microglia, clustering of T cells on microglia occurred within the first 24 hr (Figure 3), and B7/BB-1 expression was induced on the microglia (Figure 4a,b and insets), to a lesser extent than that found on IFN gamma treated controls (Figure 4c).

Inhibition of microglial antigen presentation capacity by anti-B7 antibody and CTLA-4 Ig

As previously demonstrated (10,11) enriched microglial cultures were able to present the recall antigen, tetanus toxoid protein to immediately ex-vivo autologous CD4$^+$ T cells and present antigen in a MLR system. Preincubation of microglia with anti BB-1 (10-100μg/ml) partially inhibited the primary T cell response (Table 1a,b). Pre-incubation of microglia with the fusion protein, CTLA-4 Ig (2-10 μg/ml) inhibited the ability of microglia to present antigen in both the recall assay and the mixed lymphocyte reaction, to an extent greater than the anti-BB-1 mAb (Table 1a,b). The inhibition of both the recall and primary MLR responses by microglia using the CTLA-4 Ig fusion protein was similar to the inhibition seen using the anti-HLA-DR

mAb L243 (Table 1). The anti BB-1/B7 mAb did not inhibit the ability of non-rosetting E- cells consisting of monocytes and B cells to stimulate allogeneic T cells in the MLR (39,861 $\pm$ 4,407 cpm vs. 36,660 $\pm$ 1934 cpm, n=3)).

Pre-incubation of microglia with the anti-BB-1 mAb inhibited the ability of microglia to stimulate previously activated $CD4^+$ T cells in a secondary MLR; the BB-1 mAb did not inhibit the capacity of human fetal astrocyte cultures to support the secondary MLR (Table 2). The HLA-DR blocking mAb inhibited the ability of both microglia and astrocytes to stimulate previously activated T cells in the MLR.

B7/BB-1 expression on MS brain

Immunohistochemical analysis of tissue sections from the same material used to establish enriched microglial cultures, showed these tissues to be negative for B7/BB-1 reaction product (data not shown). Within active MS lesions B7/BB-1 immunoreactivity was found in selective areas on reactive microglia. (Figure 5). These parenchymal cells were Leu-$M3^-$ and NSE^- as seen by serial sections thus discriminating these microglia from infiltrating monocytes macrophages (10,14,15). No B7/BB-1 immunoreactivity was found within non-lesion parenchymal MS brain material, though perivascular microglia and perivascular infiltrates, most likely representing T cells and infiltrating monocytes, were also positive for the reaction product (Figure 5).

DISCUSSION

Ligation of the TcR by an APC is a minimal requirement for antigen restricted T cell stimulation. Secondary signals in addition to that provided by MHC class II molecules on the APC are required for resultant T cell activation (16,17). Whether the second signals are cytokines (18,19) or cell surface adhesion molecules (20,21), or both, is not resolved. Several studies have shown that the accessory molecules including LFA-3, B7/BB-1, and ICAM-1 expressed on APC contribute critical signals to the T cells. The selective contribution of each of these molecules for primary

versus secondary T cell responses is however not yet resolved. Prior studies using mAb to block APC/T cell interactions have demonstrated that B7/BB-1 co-stimulation is required for the efficient generation of a primary MLR against alloantigen (22,23). Other studies using anti-T cell receptor antibodies and fusion proteins to stimulate naive and Ag specific T cells in the absence of APCs, have demonstrated that B7 and LFA-3 molecules function in the support of secondary T cell responses while ICAM-1 is necessary for the generation of a primary response (24,25). We have previously demonstrated that adult human derived microglia are able to present recall antigen to T cells (10), to present antigen to immediately ex-vivo $CD4^+$ T cells in a primary MLR (11), and to support the proliferation of previously sensitized T cells in a MLR. Human fetal astrocytes were able to support the continued proliferation of previously activated T cells, but can not present antigen in a primary MLR without the addition of rIL-2, rIL-1, or non-priming concentrations of microglia (Williams et al., submitted).

The microglia and astrocytes in our assay system both expressed the immune accessory/adhesion molecules ICAM-1 and LFA-3, but only microglia expressed B7/BB-1 that was upregulated following incubation with IFN gamma. B7/BB-1 expression on microglia could also be induced by the co-incubation of purified $CD4^+$ T cells with microglia, possibly the result of T cell secreted cytokines or the crosslinking of MHC class II molecules on microglia by the T cells (26). The observed expression of B7/BB-1 antigen on microglia parallel a previous report demonstrating the induction on human peripheral blood monocytes following IFN gamma incubation (4)

Functionally, we could demonstrate a partial inhibition of both recall antigen responses and antigen presentation in the MLR by pre-incubating microglia with an anti-B7/BB-1 mAb and the CTLA-4 Ig fusion protein, with more convincing numbers using the CTLA-4 Ig protein. The ability of anti B7/BB-1 mAbs to partially inhibit allogeneic T cell responses has been demonstrated using dendritic cells (27,28), EBV transformed B cells (5) or T cells (29,30) as the stimulator cells. Though activated

human peripheral blood monocytes have been demonstrated to express B7/BB-1 antigens in vitro, inhibition studies using anti B7/BB-1 mAb did not demonstrate a functional role for B7/BB-1 expression on these cells (4). The limited and variable capacity of the anti-B7/BB-1 mAb to inhibit the ability of microglia to function as an APC might be explained on the basis of the antibody affinity to the B7/BB-1 antigen. The CTLA-4 Ig fusion protein is a CD28 homologue expressed on activated T cells (5) that is also thought to bind to B7/BB-1 with a 20 fold greater affinity than the anti BB-1 mAb (5). The reported discordant expression of BB-1 and B7 on keratinocytes raises the possibility that the anti BB-1 mAb and the CTLA-4 Ig might also bind and therefore block functionally distinct molecules on the APC (31). The existence of a second B7-2 molecule that might be more important for initial T cell stimulation than B7-1 has now been described (32-34)

While we demonstrate that the antibody against B7/BB-1 and the CTLA-4 Ig fusion protein inhibited the ability of microglia to stimulate previously activated $CD4^+$ T cells, the same incubation protocol did not inhibit the ability of astrocytes, a B7/BB-1 negative cell, to support the continued proliferation of T cells. The anti-HLA-DR blocking antibody inhibited the ability of both microglia and astrocytes to present Ag to the previously activated T cells in a 2° MLR.

Human fetal astrocytes may be similar to keratinocytes that also express high levels of ICAM-1 and HLA-DR antigens following IFN gamma exposure but are B7 negative cells (31). When keratinocytes are used as stimulators in primary APC assays the activation of T cells does not occur and instead the T cells become anergic (31, 35,36).

In frozen sections of MS brain material, BB-1 antigen expression was demonstrated on activated microglia in selected lesions. We did not detect B7/BB-1 expression within adjacent non-lesion sections from MS brain or from the resected tissues from which microglial cultures were derived. B7/BB-1 expression was noted on perivascular cells and infiltrates resembling monocytes and T cells. B7/BB-1 expression in MS brain might be another indicator of cellular activation in lesion areas.

The expression of B7/BB-1 antigens in situ on frozen tissue sections has been described (37) where the authors fail to demonstrated expression on fixed tissue sections of the same medullary material (37). Fixation of tissues should therefore be considered when analyzing autopsy materials.

$CD4^+$ T cells play a critical role in the MS and EAE pathology. Rodent studies have demonstrated that activated T cells preferentially enter the CNS irrespective of MHC class restriction or antigen specificity (38). Whether T cells in the CNS are further stimulated to proliferate or instead become anergic or apoptotic (39,40) might depend upon the brain microenviroment and putative CNS APCs. Although both astrocytes (41,42) and microglia (43-44) have been implicated as putative CNS APC, it is generally considered that microglia are the most likely cell to function as a resident CNS APC (43-45). While early studies have suggested that there might exist a correlation between the induction of rodent MHC class II expression and susceptibility to EAE (46) these data have been recently questioned (47). Our data suggest that T cell immune reactivity in the CNS might be as much a result of aberrant B7/BB-1 expression as of MHC class II induction.One report has demonstrated that microglia can present antigen in vivo (45)

Our previous data demonstrate that adult human derived microglia represent a glial cell type that can present recall antigen and present antigen in a MLR to immediately ex-vivo $CD4^+$ $CD4^+$ T cells in vitro, and suggest that in vivo microglia might function to support either the initial or continued activation of T cells that cross the blood brain barrier. Our current data suggest that the expression of B7/BB-1 on microglia might be central to such $CD4^+$ T cell stimulation in the CNS.

References

1. Freedman A.S., G. Freeman, J.C. Horowitz, J. Daley, and L.M. Nadler. 1987. B7, a B cell restricted antigen that identifies preactivated B cells. J Immunol. 139:3260.

2. Yokochi T., R.D. Holly, and E.A. Clark. 1982. N lymphoblast (BB-1) expressed on epstein-barr virus-activated B cell blasts, B lymphoblastoid cell lines, and Burkitts lymphoma. J Immunol. 128:823.

3. Young J.W., L.Koulova, S.A. Soergel, E.A. Clark, R.M. Steinmann, and B. Dupont. 1992. The B7/BB-1 antigen provides one of several costimulatory signals for the activation of $CD4^+$ T lymphocytes by human blood dendritic cells in vitro. J Clin Immunol. 90:229.

4. Freedman A.S., G.J. Freeman, K. Rhynhart, and L.M. Nadler. 1991. Selective induction of B7/BB-1 on interferon gamma stimulated monocytes: a potential mechanism for amplification of T cell activation through the CD28 pathway. Cell Immunol. 137:427

5. Linlsley P.S., E.A Clark, and J.A. Ledbetter. 1990. T cell antigen CD28 mediates adhesion with B cells by interacting with activation antigen B7/BB-1. Proc Natl Acad Sci (USA). 87:5031

6. Hara T., S. M. Fo, and J.A. Hanses. 1985. Human T cell activation. II a new activation pathway used by a major T cell proliferation via a disulfide-bonded homodimer of a 44-kilodalton polypeptide (9.3 antigen). J Exp Med. 161:1513.

7. Linsley P.S., W. Brady, M. Urnes, L.S. Grosmaire, N.K. Damle, and J.A. Ledbetter. 1991. CTLA-4 is a second receptor for the B cell activation antigen B7. J Exp Med. 174:561.

8. Fraser J.D., B.A. Irving, G.R. Crabtree, and A. Weiss. 1991. Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. Science. 251:313.

9. Lindstein T., C.A. June, J.A. Ledbetter, G. Stella, and C.B. Thompson. 1989. Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. Science. 244:339.

10. Williams K., A. Bar-Or, E. Ulvestad, A. Olivier, J.P. Antel, and V.W. Yong. 1992. Biology of adult human microglia in culture: comparisons with peripheral blood monocytes and astrocytes. J Neuropath Exp Neurol. 51:538.

11. Williams K., E. Ulvestad, L. Cragg, M. Blain, and J.P. Antel. 1993. Induction of primary T cell responses by human glial cells. J Neurosci Res. 36:382.

12. Yong V.W. and J.P. Antel. 1992. Culture of glial cells from human biopsies. In A. Richardson and S. Fedoroff (eds): Protocols for neural cell culture. St. Louis:Humana Press, 81.

13. Pellegrino M.A., S. Ferrone, and A.N. Theofilopoulos. 1976. Isolation of human T and B lymphocytes by rosette formation with 2-aminoethylisothioquronium bromide (AET)- treated sheep red blood cells. J Immunol Meth. 11:273.

14. Ulvestad E., K. Williams, R. Matre, H. Nyland, A. Olivier, and J.P. Antel. 1993. Fc Receptors for IgG on cultured human microglia mediate cytotoxicity and phagocytosis of antibody-coated targets. J Neuropath Exp Neurol. 53 (In press).

15. Ulvestad E., K.Williams, C. Vedler, J. Antel, H. Nyland, S Morke, and R. Matre. Reactive microglia in multiple sclerosis lesions have an increased expression

of receptors for the Fc part of IgG. J Neurolog Sci (In press).

16. Mueller D.L., M.K. Jenkins, and R.H. Schwartz. 1989. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. Ann Rev. Immunol. 7:445.

17. Schwartz R.H. 1990. A cell culture model for clonal anergy. Science. 248:1349.

18. Kurt-Jones E.A., D.I. Beller, S.B. Mizel, and E.R. Unanue. 1985. Identification of a membrane-associated interleukin 1 in macrophages. Proc Natl Acad Sci (USA) 82:1204.

19. Koide S., and R.M. Steinman. 1989. Induction of interleukin 1α mRNA during the antigen-dependent interaction of sensitized T lymphoblasts with macrophages. J Exp Med. 168:409.

20. Springer, T.A. 1990. Adhesion receptors of the immune system. Nature 346:425.

21. Steinman, R.M. and J.W. Young. 1991. Signals arising from antigen presenting cells. Curr. Opin. Immunol. 3:361.

22. Azuma M., M. Cayabyab, D. Buck, J.H. Philips, and L.L. Lanier. 1992. CD28 interactions with B7 costimulated primary allogeneic proliferative responses and cytotoxicity mediated by small, resting T lymphocytes. J. Exp. Med. 175:353.

23. Norton S.D., L. Zuckerman, K.D. Urdahl, R. Shefner, J. Miller, and M.K. Jenkins. 1992. The CD28 ligand, B7, enhances IL-2 production by providing costimulatory signal to T cells. J. Immunol. 149:1556.

24. Damle N.K., K. Klussman, P.S. Linsley, and A. Arrufo. 1992. Differential costimulatory effects of adhesion molecules B7, ICAM-1, LFA-3, and VCAM-1 on resting and antigen-primed $CD4^+$ T lymphocytes. J. Immunol. 148:1985.

25. Damle N.K., K. Klussman, P.S. Linsley, A. Aruffo, and J.A. Ledbetter. 1992. Differential regulatory effects of intercellular adhesion molecule-1 on costimulation by the CD28 counter-receptor B7. J. Immunol. 149:2541.

26. Nabavi N., G.J. Freeman, A. Gault, D. Godfrey, L.M. Nadler, and L.H. Glimcher. 1992. Signalling through the MHC class II cytoplasmic domain is required for antigen presentation and induces B7 expression. Nature 360:266.

27. Symington F.W., W. Brady, and P.S. Linsley. 1993. Expression and function of B7 on human epidermal langerhan cells. J Immunol. 150:1286.

28. Koulova L., E.A. Clark, G. Shu, and B. Dupont. 1991. The CD28 ligand B7/BB-1 provides costimulatroy signal for alloactivation of $CD4^+$ T cells. J Exp Med. 173:759.

29. Azuma M., H. Yessel, J.H. Phillips, H. Spits, and L.L. Lanier. 1993. Functional expression of B7/BB-1 on activated T lymphocytes. J Exp Med. 177:845.

30. Wyss-Coray T., D. Mauri-Hellweg, K. Baumann, R. Grunow, and W.J. Pichler. 1993. The B7 adhesion molecule is expressed on activated human T cells: functional involvement in T-T cell interactions. Eur J Immunol. 23:2175.

31. Nickoloff B.J., R.S. Mitra, K. Lee, L.A. Turka, J. Green, C. Thompson, and Y. Shimizu. 1993. Discordant expression of CD28 ligands, BB-1 and B7 on keratinocytes in vitro and psoriatic cells in vivo. Am J Pathol. 142:1029.

32. Hathcock K.S., G. Laslo, H.B. Dickler, J. Bradshaw, P. Linsley, and R.J. Rhodes. 1993. Identification of an alternative CTLA-4 ligand costimulatory for T cell activation. Science. 262:905.

33. Freeman G.J., F. Borriello, R.J. Rhodes, H Reiser, K.S. Hathcock, G. Laszo, A.J. McKnight, J. Kim, L Du, D.B. Lombard, G.S. Gray, L.M. Nadler, and A. H. Sharpe. 1993. Uncovering of functional alternative CTLA-4 counter-receptor in B7-deficient mice. Science 262:907.

34. Freeman G.J., J.G. Gribben, VA. Boussiotis, J.W. Ng, V.A. Restivo, L.A. Lombard, G.S. Gray, and L.M. Nadler. 1993. Cloning of B7-2: a CTLA-4 counter-receptor that costimulates human T cell proliferation. Science 262:909.

35. Gaspari A.A, M.A. Jenkins, and S.I. Katz. 1988. Class II MHC-bearing keratinocytes induce antigen-specific unresponsiveness in hapten-specific TH1 clones. J Immunol. 141:2216.

36. Bal V., A. McIndoe, G. Denton, D. Hudson. G. Lombardi, J. Lamb, and R. Lechler. 1990. Antigen presentation by keratinocytes induces tolerance in human T cells. Eur J Immunol. 20:1893.

37. Nelson A.J., S. Hosier, W. Brady, P.S. Linsley, and A.G. Farr. 1993. Medullary thymic epithelium express a ligan for CTLA4 in situ and in vitro. J Immunol. 151:2453.

38. Hickey W.F., B.L. Hsu, and H. Kimura. 1991. T-lymphocytes entry into the central nervous system. J Neurosci Res. 28:254.

39. Pender M.P., P.A. McCombe, G. Young, and K.B. Nguyen. 1992. Apoptosis of

αß T lymphocytes in the nervous system in experimental autoimmune encephalomyelitis: its possible implication for recovery and acquired tolerance. J Autoimmun. 5:401.

40. Schmied M., H. Breitschopf, R. Gold, H. Zischler, G. Rothe, H. Wekerle, and H. Lassmann. 1993. Am J Pathol. 143:446.

41. Traugott U., L.L. Scheinberg, and C.S. Raine. 1985. On the presence of Ia-positive endothelial cells and astrocytes in multiple sclerosis and its relevance to antigen presentation. J Neuroimmunol. 8:1.

442. Lee S.C., G.R.W. Moore, G. Golenwsky, and C.S. Raine. 1990. Multiple sclerosis: a role for astroglia in active demyelination suggested by class II MHC expression and ultrastructural study. J Neuropath Exp Neurol. 49:122.

43. Hayes G.M., M.N. Woodroofe, and M.L. Cuzner. 1987. Microglia are the major cell types expressing MHC class II in human white matter. J Neurol Sci. 80:25.

44. Matsumoto Y., N. Hara, R. Tanka, and M. Fujiwara. 1986. Immunohistochemical analysis of the rat central nervous system during experimental allergic encephalomyelitis with special reference to Ia-positive cells with dendritic morphology. J Immunol. 136:3668.

45. Hickey W.F. and H. Kimura. 1988. "Perivascular microglia" are bone marrow derived and present antigen in vivo. Science 239:290.

46. Massa P.T., V. ter Meulen, and A. Fontana. 1987. Hypeinducibility of Ia antigen on astrocytes correlates with strain-specific susceptibility to experimental autoimmune encephalomyelitis. Proc Natl Acad Sci (USA). 84:4219.

47. Sedgwick J.D.,S. Schwender, R. Gregersen, R. Dorries, V. t Meulen. 1993. Resident macrophages (ramified microglia) of the adult brown norway rat central nervous system are constituitively major histocompatability complex class II positive. J Exp Med. 177:1145.

Table 1. Inhibition of the antigen presentation capacity of adult human microglia with anti BB-1 mAb, and the CTLA-4 Ig fusion protein

Antigen presentation assay		% Inhibition	
	Blocking Ab	Mean	Range
Recall antigen	anti BB-1	37%	19-55% (n=8)
	CTLA-4	51%	20-67% (n=8)
	anti-HLA-DR	67%	56-72% (n=3)
MLR	anti BB-1	22%	15-42% (n=6)
	CTLA-4	73%	62-77% (n=3)
	anti-HLA-DR	71%	65-76% (n=3)

Table 2. Anti- BB-1 mAb inhibits the ability of microglia to support the continued proliferation of previously activated $CD4^+$ T cells in a MLR

		% Inhibition Mean	
Microglia	anti BB-1	59%	(n=2)
	anti HLA-DR	62%	(n=2)
Astrocytes	anti BB-1	21%	(n=2)
	anti HLA-DR	61%	(n=2)

Figure Legends

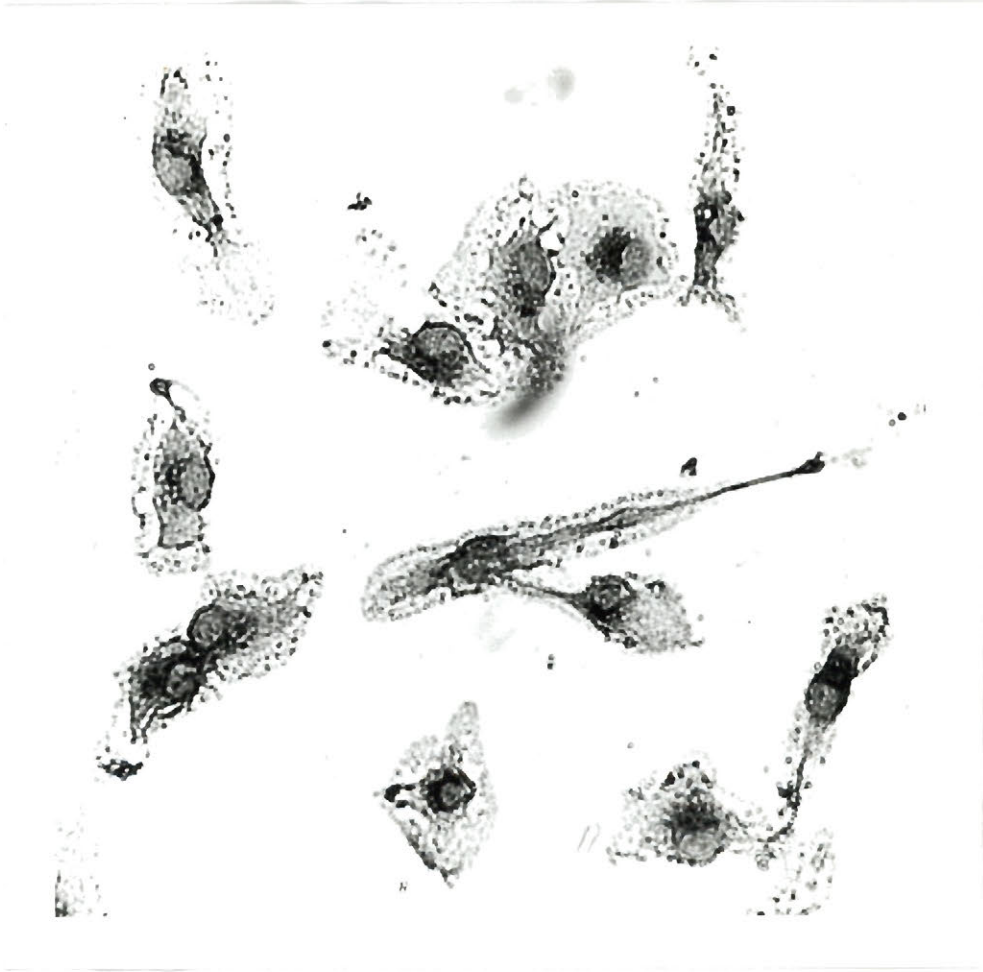
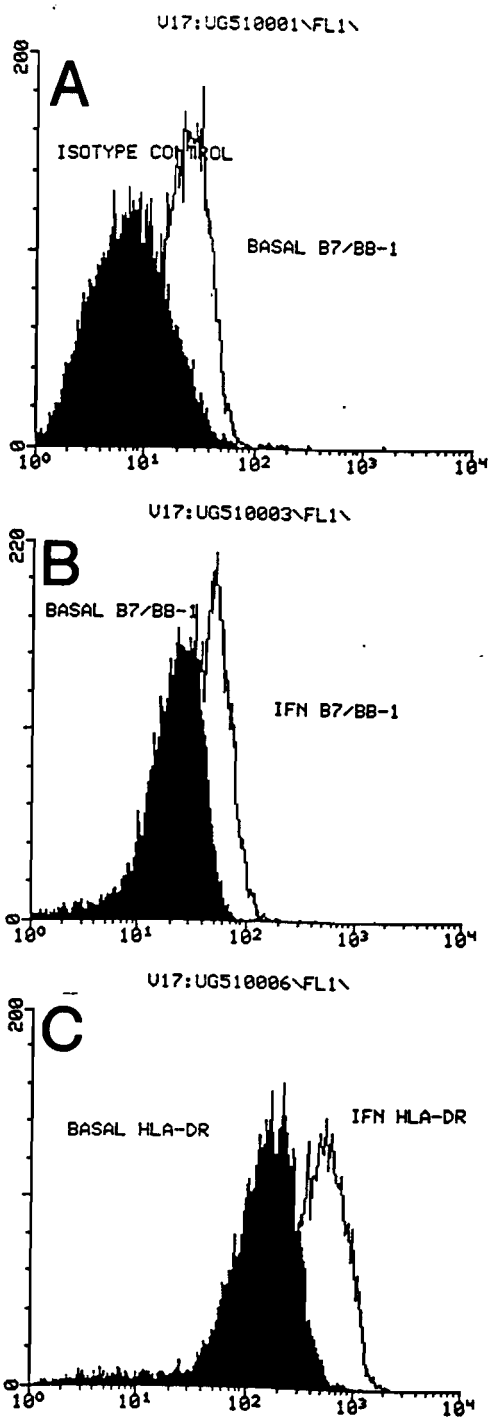


Figure 1. Adult human derived microglia in vitro (enriched cultures >95% purity). Leu-M5 immunoreactivity was demonstrated by fixing cells with ice-cold acetone for 5 min followed by incubation of Leu-M5 mAb overnight. A biotinylated rabbit anti-mouse immunoglobulin (1:300) was then incubated for 30 min followed by a avidin-biotin-peroxidase complex (ABComplex/HRp) for 30 min. The colored reaction product was developed using 3-amino-9-ethyl-carbazole containing buffer (300X mag).

Figure 2. B7/BB-1 and HLA-DR antigen expression on microglia in vitro. Microglia were stained either with anti-BB-1 mAb followed by a goat-anti- mouse FITC or with a FITC labelled anti-HLA-DR mAb. Histograms represent 10,000 events analyzed showing A) basal B7/BB-1 expression of enriched microglial cultures over an isotype control. B) B7/BB-1 on non stimulated microglia and microglia that had been incubated with IFN gamma (100 U/ml, 24 hr) prior to staining. C) Basal and IFN gamma stimulated HLA-DR expression on microglia.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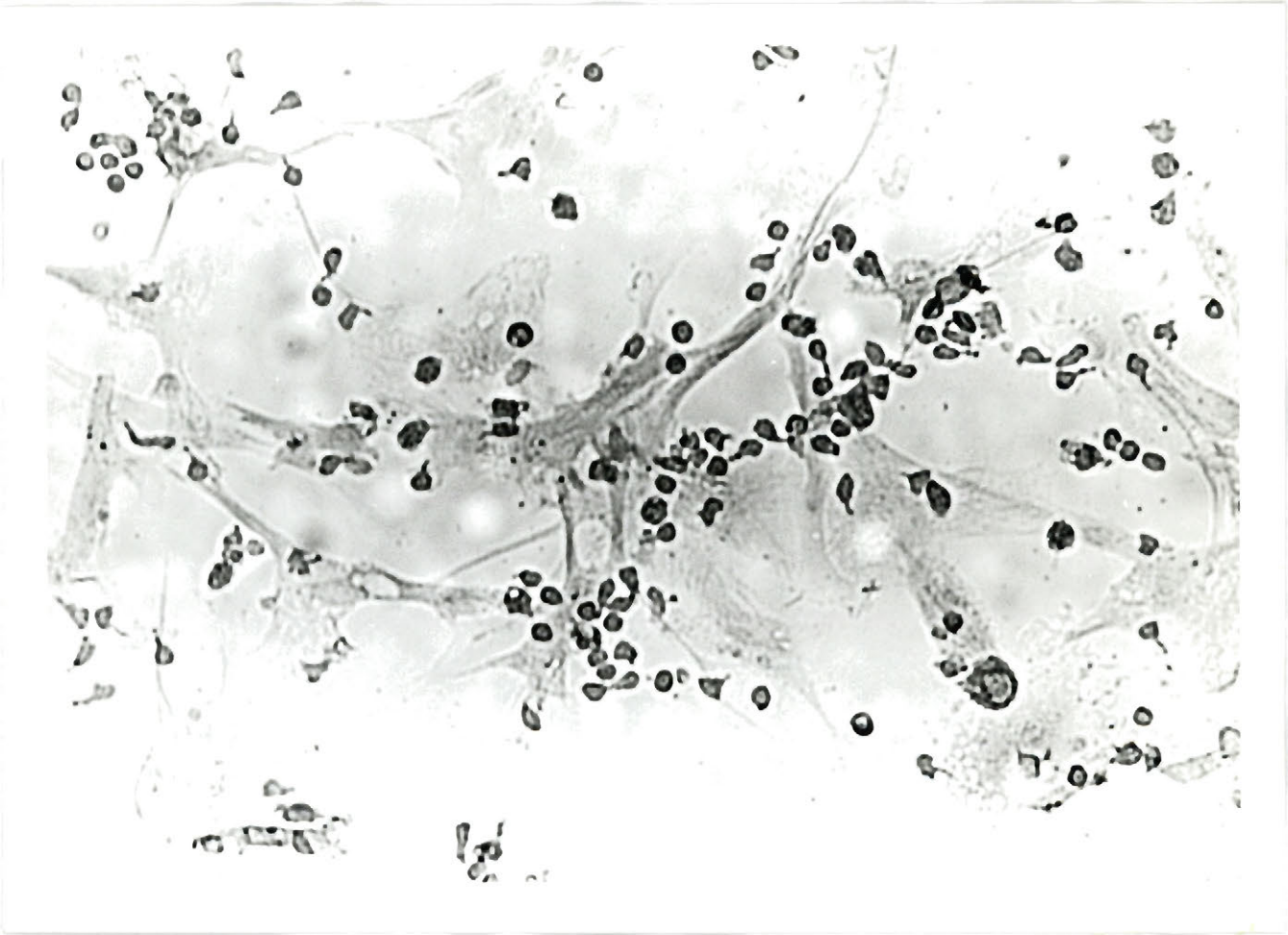

Figure 3. Co-culture of $CD4^+$ T cells with microglia results in clustering of T cells around microglia. $1X10^3$ microglia were seeded onto poly-l-lysine coated coverslips and placed into a 30 mm^3 culture dish with 3 ml of culture medium. $CD4^+$ T cells ($1x10^5$ cells per ml) were co-cultured with microglia for 24 hr. Coverslips were briefly dipped in PBS (3X), and cells were fixed with 4% paraformaldehyde, and examined using a phase contrast microscope (300X mag).

Figure 4. B7/BB-1 expression on microglia is induced with IFN gamma or by co-culturing microglia with $CD4^+$ T cells. A) $1X10^3$ microglia were seeded onto coverslips and co-cultured with $CD4^+$ T cells or IFN gamma (100 U/ml for 24 hr) and compared with sister microglia cultures that were cultured without T cells (inset) (300X mag). B) Same field as A demonstrating B7/BB-1 expression on microglia co-cultured with $CD4^+$ T cells over that of non treated microglia (inset, same field as above inset A) (300 X mag). C) Incubation of microglial cultures with IFN gamma for 24 hr induces B7/BB-1 antigens (300x mag)

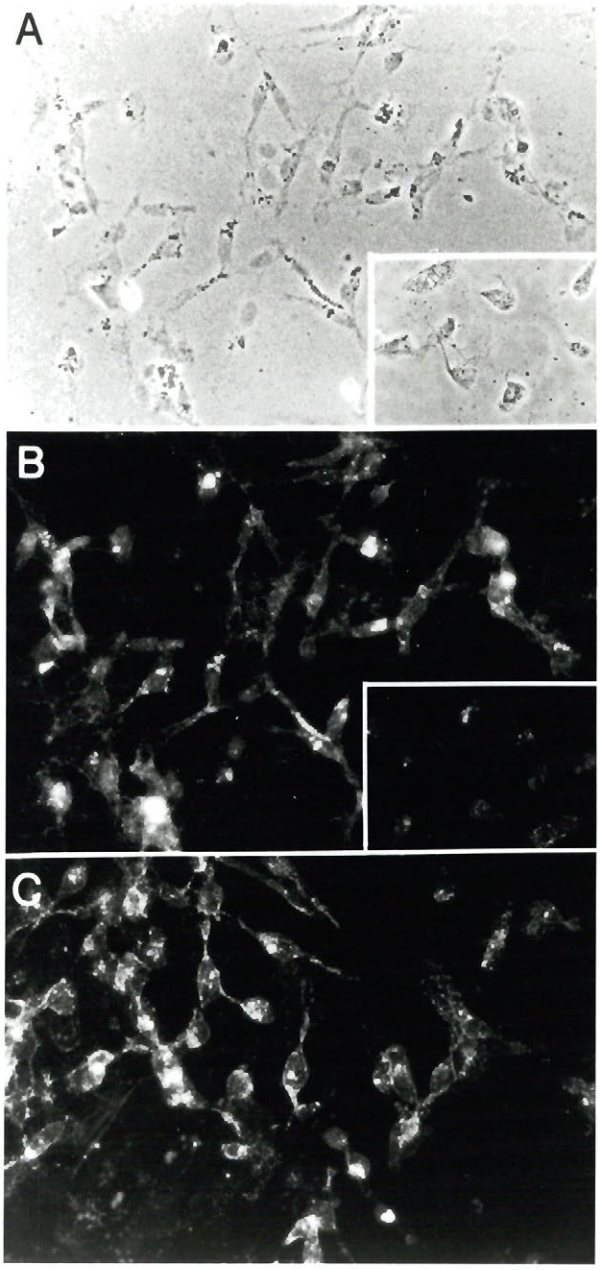
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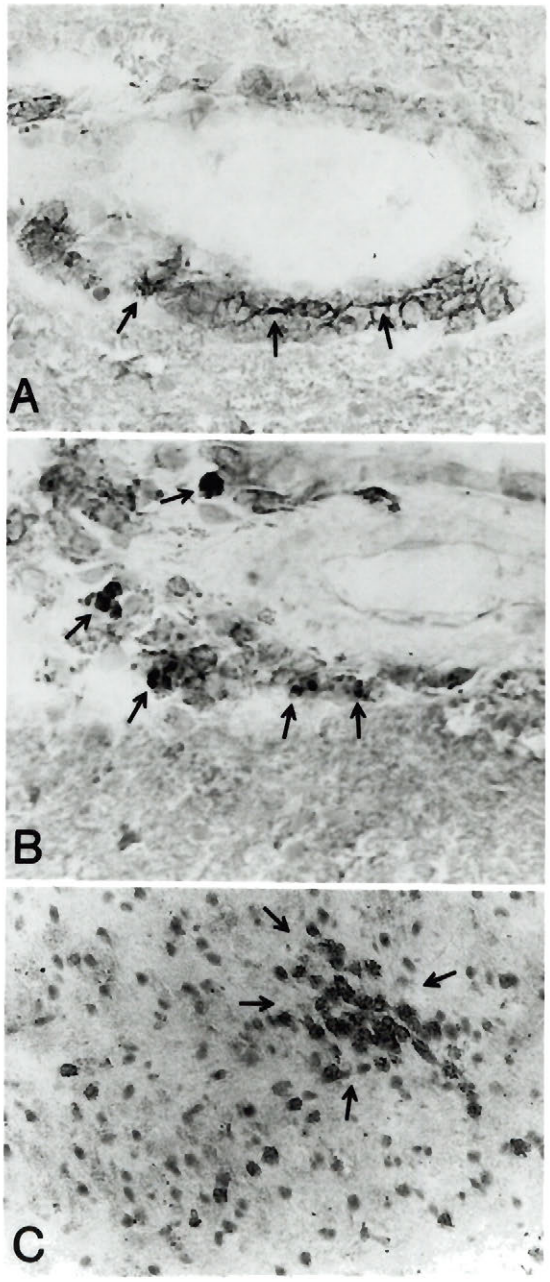
Figure 5. Immune peroxidase demonstration of B7/BB-1 on frozen tissue sections from MS brain. A) Reaction product on the surface of perivascular cells (arrows) (300X mag) B) Reaction product on infiltrating cells in perivascular cuff (arrows) (300X mag). C) Reaction product on reactive microglia in the parenchyma of an active MS lesion (300X mag).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Part III

Immune effector functions

Chapter 7

Fc RECEPTORS FOR IgG ON CULTURED HUMAN MICROGLIA MEDIATE CYTOTOXICITY AND PHAGOCYTOSIS OF ANTIBODY-COATED TARGETS

Elling Ulvestad, Kenneth Williams, Roald Matre,
Harald Nyland, Andre Olivier, Jack Antel

Preface

Following the observation of FcRs I-III on microglia in vitro (Chapter 1) the functional significance of FcR expression on microglia was evaluated including the ability of microglia to bind, phagocytose, and lyse opsonized and non-opsonized target cells. Activation of microglia as a consequence of FcR ligation was determined using an OKT3 T cell assay that is dependent upon cytokine production by the FcR expressing cell, and by assessing the oxidative burst response of microglia by the reduction of nitroblue tetrazolium. Major points demonstrated in this chapter include:

1. Confirmation of FcR I-III on microglia in vitro. Astrocytes and oligodendrocytes were FcR negative. FcR expression was also demonstrated on microglia in situ on the same resected material from which glial cell cultures were established.

2. Opsonized human red blood cells bind to microglia, and are phagocytosed and lysed, while non-opsonized cells are not. The interaction of opsonized red blood cells and microglia via FcRs result in oxidative burst by microglia.

3. Cross linking Fc receptors on microglia using an OKT3 mitogen assay results in cytokine production by microglia as demonstrated by the proliferation of peripheral blood derived T cells. Pre-incubating microglia with IFN to upregulate FcR expression results in an increased T cell response.

ABSTRACT

We have utilized surgically resected human central nervous system (CNS) tissue to determine the expression and functions of Fc receptors (FcγR) on individual cell types found within the CNS. We observed all three classes of FcγR on microglial cells in situ and in vitro, but not on astrocytes or oligodendrocytes. Incubation of cultured microglia with immune complexes (antibody-coated red blood cells) induced phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC), and oxidative bursts. We also found that microglia have the capability to produce T cell stimulatory soluble mediators after FcγR crosslinking. These functional responses were enhanced by pre-treatment of the microglia with IFN-γ. Our results implicate microglial effector responses triggered by interaction of FcγR with opsonized antigens as potential mediators of tissue injury within the CNS. Such injury may be particularly applicable to multiple sclerosis, an inflammatory demyelinating disease characterized by intrathecal production of immunoglobulins and cytokines.

INTRODUCTION

Although concealed by the blood-brain barrier, inflammatory responses are elicited in the central nervous system (CNS) against both autoantigens and infectious agents. The microglia and astrocyte cell populations of the CNS have both been implicated as regulators of immune reactivity and effectors of tissue injury within the CNS (1-3).

Development of a specific immune response within the CNS requires communication between glial cells and cells of the immune system. Expression of major histocompatibility complex (MHC) molecules on glial cells provide one type of interactive molecules. IgG Fc receptor (FcγR) expression on glial cells provide a class of surface molecules which will further promote glial-immune interactions. FcγR have been demonstrated on ramified microglia in both rat (4) and human CNS (5,6), and on the surface of microglial lipophages in multiple sclerosis (MS) lesions (7). Microglia

and macrophages phagocytose myelin in active MS lesions, apparently with IgG serving as ligand between myelin and FcγR on the phagocytosing cell (8).

The cerebrospinal fluid of MS patients characteristically contains oligoclonal IgG antibodies, some of which are reactive with myelin or its cell of origin, the oligodendrocyte (9). Experimental data obtained from rats indicate that such antibodies may be of pathogenetic significance in demyelination, probably due to antibody-dependent cell-mediated cytotoxicity (ADCC) (10).

In the present study, we have utilized surgically derived human CNS tissue to study expression of FcγR on isolated neurons and glial cells, microglia, astrocytes and oligodendrocytes. We also investigated whether binding of IgG to FcγR on isolated microglia, the glial cell on which FcγR expression was detected, induced functional activity including phagocytosis and ADCC. Since oxygen radicals can induce oligodendrocyte cell death in vitro (11) and brain injury in vivo (12), we examined whether microglia produce reactive oxygen species in response to FcγR triggering. Additional studies were performed to document that crosslinking of FcγR on microglia could promote synthesis and secretion of soluble T cell stimulatory mediators. The T cell derived cytokine IFN-γ was introduced in some experiments, following previous studies indicating that IFN-γ may potentiate antibody-mediated demyelination (10) and promote clinical exacerbations and lesion growth in MS (13,14).

MATERIALS AND METHODS

Tissue

CNS tissue was removed from seven patients undergoing surgical procedures for intractable epilepsy. Mean age of patients (four female, three male) was 38 years (range 19 - 54 years). Histological examination of tissue revealed mild to severe gliosis. Tissue was removed by Cavitron ultrasonic aspiration from either the temporal

lobe, hippocampus, or from amygdala. Tissue was dissected into multiple pieces 2-3 mm^3 in size, providing the material used to establish CNS cell cultures. Small specimens of the same tissue were blocked in Cryomount (Histolab, Gøteborg, Sweden), frozen in isopentane precooled in liquid nitrogen, and sectioned (4-6 μm) on a cryostat.

Cell culture

Isolated cells from adult tissue were obtained by a previously described protocol (15). In brief, aspirated tissue was washed extensively in PBS and dissociated with 0.25% trypsin and 20 μg/ml DNAse (GIBCO Canada, Burlington, Ont.) for 1 h at 37°C. The cell suspension was then centrifuged in 30% Percoll (Pharmacia LKB, Uppsala, Sweden) for 30 min at 4°C. Glial cells were recovered from the layer of floating cells between the top layer of myelin and the bottom layer of red blood cells. The cells were washed twice in PBS, counted, and resuspended in Eagle's minimum essential medium supplemented with 5% FCS, gentamicin 20 μg/ml, glucose 1 mg/ml, and 2 mM glutamine (GIBCO), henceforth referred to as culture medium. The cells were then either seeded onto 25 cm^2 culture flasks (Nunc, Roskilde, Denmark) or directly onto 9 mm Aclar fluorocarbon plastic cover slips (S. Kim, Vancouver, Canada) previously coated with 10 μg/ml poly-L-lysine (Sigma, St. Louis, MO) at 10^4 cells per cover slip. The cells in the culture flasks were left undisturbed over night at 37°C in a humified incubator with 5% CO_2. The following day culture flasks were lightly shaken and floating cells, mainly oligodendrocytes, were removed and seeded onto cover slips. Remaining cells, microglia and astrocytes, were then either retrypsinized and seeded onto cover slips as mixed microglia-astrocyte cultures, or were allowed to differentiate morphologically for 1 week in culture flasks. After 1 week, the microglia-astrocyte flasks were subjected to 5 hours of 150 rpm shaking on an orbital shaker. The less adherent astrocytes floated off, and the remaining cells

(>95% Leu-M5$^+$ cells) were then trypsinized and either seeded into microplate wells at 10^3 - 10^5 cells per well or onto coverslips at a density of 10^4 cells per coverslip. In some experiments glial cells were stimulated with 100 U IFN-γ (UBI, Lake Placid, NY) for 24 - 48 hours before being analyzed in the assays described below.

Isolation of monocytes and T cells

Mononuclear cells were obtained by Ficoll-Hypaque (Pharmacia LKB) density centrifugation of heparinized venous blood samples from adult donors, suspended in culture medium, and then allowed to adhere to petri dishes for 60 min at 37°C. Non-adherent cells were removed by gentle washings in warm PBS. Adherent cells were gently scraped off with a rubber policeman, and thereafter seeded onto cover slips at 10.000 cells per cover slip. Cells on cover slips were flooded one day later (16). Cells were more than 90% monocytes as determined by non-specific esterase staining and immunostaining with Leu-M3.

T cells were purified from non-adherent cells by rosetting with aminoethyl-isothiouroniumbromide hydrobromide (AET)-treated sheep erythrocytes and centrifugation upon Ficoll-Hypaque density gradients (17). Contaminating erythrocytes were lysed with a buffered ammonium chloride solution, and residual monocytes eliminated by a further adherence to plastic for 1 h at 37^{0}C. Purity of T cells was >95% as determined by immunostaining with OKT3 mAb.

Antibodies

The mAbs used in this study are summarized in Table 1. MAbs 32.2 and 3G8 were used as F(ab')$_2$ fragments and IV.3 as Fab fragments. Biotinylated rabbit anti-mouse immunoglobulins were obtained from Dakopatts, Glostrup, Denmark. Rhodamine-conjugated goat anti-mouse immunoglobulins, fluorescein-conjugated goat anti-rabbit immunoglobulins and F(ab')$_2$ fragments of affinity purified goat anti-mouse

IgG were obtained from Cappel, West Chester, PA.

WinRho human IgG anti-D was obtained from Winnipeg Rh Institute Inc., Winnipeg, Canada. IgG was digested with pepsin (Sigma) to prepare $F(ab')_2$ fragments (18). Immune complexes of horseradish peroxidase (HRP) and rabbit IgG anti-HRP or rabbit $F(ab')_2$ fragments of IgG anti-HRP were prepared as described (19). MAbs and secondary antibodies were diluted in Hanks' balanced salt solution containing 10% normal goat serum, 2% horse serum, and 1mM Hepes buffer.

Immunofluorescence analysis

Mixed astrocyte-microglia cultures on cover slips were analyzed by indirect double immunofluorescence labelling techniques in order to permit evaluation of both cell type and expression of FcγR. Viable unfixed cells were incubated with murine mAbs for two hours at 4^0C, and thereafter with rhodamine-conjugated goat anti-mouse antibodies diluted 1:100 for 45 min at 4^0C. Following fixation with 95% ethanol-5% glacial acetic acid mixture at -20^0C for 15 min, antibodies to GFAP were introduced for 45 min at room temperature. Fluorescein conjugated goat anti-rabbit immunoglobulins diluted 1:100 were then applied for 45 min at room temperature. All incubations were followed by washings in PBS. Cover slips were finally mounted on glass slides with Gelvatol (Air Products and Chemicals Inc, Allentown, PA). Enriched oligodendrocyte cultures were assessed for FcγR expression using single immunostaining with anti-FcγR mAbs.

Negative controls consisted of sister cultures in which the primary antibodies were omitted. A Leitz microscope equipped with rhodamine and fluorescein optics was used for immunofluorescence analysis.

Phosphatidylinositol phospholipase-C treatment

Phosphatidylinositol phospholipase C (PI-PLC), an enzyme that hydrolyzes PI

anchors, was obtained from Boehringer Mannheim, Mannheim, Germany. PI-PLC was diluted in PBS. Microglia were treated with 0.1 U/ml PI-PLC at 37°C for 60 min (20). They were thereafter washed in PBS, and immunostained with 3G8 or 1F5 as described above. Cultures incubated in PBS without PI-PLC were used as controls for PI hydrolysis.

Non-specific esterase activity

Non-specific esterase (NSE) was detected using reagents obtained from Sigma (21). Cells on cover slips were fixed at 4°C in a mixture of 2% glutaraldehyde/60% acetone for 5 min, rinsed with distilled water, and air dried for 15 min. A mixture of pararosaniline solution, sodium nitrate, and alpha-naphthyl butyrate was then employed for 1 h at 37°C. Cells were thereafter rinsed with distilled water, air dried, and mounted. Positive cells were scored using a light microscope.

Immunoperoxidase staining

Cryostat sections from adult brain tissue were fixed in ice cold acetone for 5 min. Sections were washed in PBS and incubated over night at 4°C in a humid chamber with mAbs diluted in PBS with 10% normal rabbit serum. Biotinylated rabbit anti-mouse immunoglobulins diluted 1:300 were applied for 30 min. Avidin-biotin-peroxidase complex (ABComplex/HRP, Dakopatts code K 355) was prepared as recommended by the manufacturer, and allowed to react with sections for 30 min. Sections were thereafter washed in PBS and treated with a 3-amino-9-ethyl-carbazole containing buffer for the development of a colored reaction product. Controls consisted of similarly treated sections in which primary mAbs were omitted. Sections were finally counterstained with hematoxylin, mounted in Glycergel (Dakopatts), and analyzed in a light microscope.

Phagocytosis assay

This assay was a modification of the assay described by Newman et al. (22). Human O Rhesus D+ erythrocytes (E) were isolated from citrated blood collected from healthy laboratory personnel. E were washed in PBS and adjusted to 2 x 10^8 E/ml. 10^7 E in 100 μl PBS were incubated with an equal volume of IgG anti-D or F(ab')$_2$ fragments of IgG anti-D for 60 min at 37^{0}C. After washings in PBS, IgG labelled E (EA), IgG F(ab')$_2$ labeled E (EA-F(ab')$_2$) and E were resuspended in growth medium.

Cover slips with 10^4 microglia were incubated with 5 x 10^6 EA, EA-F(ab')$_2$ or E at 37^{0}C. After 30 min, cover slips were gently washed in cold PBS. For the determination of rosette-forming cells, cells were immediately fixed in 4% glutaraldehyde in PBS. Rosette forming cells were thereafter counted using a light microscope. Results are presented as number of rosetting cells/100 cells. For determination of phagocytosis, adherent non-phagocytosed EA were lysed after a brief dip in distilled water. Cells were thereafter immediately transferred back to PBS before fixation in 4% glutaraldehyde. 100 microglia were counted, and number of phagocytosing microglia were determined. The phagocytic index is the number of EA ingested/microglial cell. All results are presented as mean values of triplicate experiments. For blocking studies, microglia were incubated for 30 min at 4^{0}C with immune complexes before addition of EA.

ADCC assay

Cytotoxicity assays were performed in U-bottomed microtiter plates (Costar, Cambridge, MA) (23). 10^5 microglia/well were cultured overnight in 100 μl medium. 10^8 E were incubated with 100 μCi ^{51}Cr (sodium chromate, Radiochemical Center, Amersham, UK) at 37^{0}C for 30 min. E, EA, and EA-F(ab')$_2$ cells were then prepared as for the phagocytosis assay. 10^6, 5 x 10^5 and 10^5 ^{51}Cr-labeled EA, EA-F(ab')$_2$ or E

in 100 μl medium were thereafter added to wells. Plates were centrifuged (2 min, 500 x g) at room temperature and incubated for 150 min in a humified incubator at 37^{0}C. Plates were then centrifuged (10 min, 400 x g, room temperature), and the supernatant was removed for estimation of ^{51}Cr release (experimental release, ER). 100 μl 5N NaOH was added to induce maximal ^{51}Cr release (M). Spontaneous release (S) was determined by the incubation of EA without effector cells as well as by incubation of E with effector cells. Spontaneous release did not exceed 3% of the maximal release. All experiments were performed in triplicate. ^{51}Cr release was estimated by an automatic Pharmacia LKB gamma counter. Specific killing was calculated according to the equation (ER - S)/(M - S) x 100. For blocking studies, microglia were incubated for 30 min at 4^{0}C with immune complexes before addition of EA.

Oxidative burst assay

Nitroblue tetrazolium (NBT) was purchased from Sigma. NBT acts as an unspecific electron acceptor in biochemical pathways activated during the oxidative burst, producing a blue precipitate within the reactive cell (24). NBT was dissolved in 0.9% NaCl at 1 mg/ml. Phorbol myristate acetate (PMA) (Sigma), a potent activator of protein kinase C, was used at a final concentration of 500 ng/ml. Microglia on cover slips were incubated with 30 μl NBT and either 20 μl PMA, EA, EA-F(ab')$_2$ or E. After 45 minutes incubation at 37^{0}C in 5% CO_2, uningested EA were lysed in distilled water. Cells were fixed in 4% glutaraldehyde and mounted. Microglia stimulated with mAbs, were preincubated for 30 minutes at 4^{0}C with either 32.2, IV.3, 3G8, a mixture of 32.2, IV.3 and 3G8, or Leu-M5, all diluted 1:10. After washing in PBS, 30 μl NBT was added together with 20 μl F(ab')$_2$-fragments of goat anti-mouse IgG diluted 1:100. After 45 minutes incubation at 37^{0}C, cells were washed in PBS, fixed in glutaraldehyde and mounted. Coverslips were examined in a light microscope, and results are presented as percent cells with reduced NBT in their

cytoplasm. 100 cells were counted per coverslip. All experiments were performed in triplicate.

OKT3-induced T cell proliferation

The assay was performed as described (25), with slight modifications. 1000 microglia were co-cultivated with 10^5 allogeneic T cells and OKT3 mAb at 2.5 ng/ml for three days in flat-bottomed 96 well microtiter plates (Costar). To quantitate the degree of DNA synthesis, cultures were pulsed with 1 μCi [^{3}H]-thymidine (ICN Biomedicals, Mississauga, Ontario) per well for 5 hours and harvested onto glass filters with a Titertek Cell Harvester 550 (Flow Laboratories Inc., Mississauga, Ontario). Radioactive counts were measured on a Pharmacia LKB Beta liquid scintillation counter and were expressed as counts per minute (CPM). All experiments were performed in triplicate.

Statistics

The Mantel-Haenszel chi-square test and a two sample t-test were used for statistical evaluations. A two sided P value of 0.05 or less was considered significant.

RESULTS

Characterization of neural cells

Mixed glial cell-cultures from adult donors consisted of microglia (Leu-M5$^+$), astrocytes (GFAP$^+$), and oligodendrocytes (GalC$^+$). Morphologic differentiation of the cells was evident after seven days in culture. Oligodendrocytes and astrocytes had well-developed processes radiating from the cell bodies, whereas most of the microglia had developed into bipolar cells. Microglia could be differentiated from astrocytes and oligodendrocytes on the basis of intracellular autofluorescent granula. The auto-

fluorescence varied between different cell preparations. In contrast to blood-derived monocytes, microglia were not stained by non-specific esterase or Leu-M3.

In CNS tissue sections, numerous microglia reactive with Leu-M5 were observed throughout the parenchyma. Leu-M3 and NSE positive cells were infrequent. Such cells were not detected in the parenchyma, but were exclusively located in close proximity to blood vessels.

FcγR expression on neural cells

In vitro - Cells reactive with mAbs to FcγRI, FcγRII, and FcγRIII were identified in the mixed adult-derived astrocyte-microglia cultures from all donors (Fig 1). All FcγR immunoreactive cells were GFAP negative. Double staining with immune complexes and Leu-M5 verified that all FcγR positive cells were also Leu-M5 positive. No FcγR mAb reactivity was observed on enriched oligodendrocyte cultures. Pre-treatment of cultures with IFN-γ for 24-48 hours did not induce FcγR expression on astrocytes or oligodendrocytes. CD59 reactivity on microglia was strongly reduced after PI-PLC treatment, whereas no difference in FcγRIII expression could be observed between PI-PLC treated and untreated microglia. No staining was observed when primary mAbs were omitted.

In situ - The mAbs 32.2 (FcγRI), IV.3 (FcγRII) and 3G8 (FcγRIII) stained cells scattered in the parenchyma (Fig 2). The staining intensity of anti-FcγR mAbs was weaker than the staining with Leu-M5. Fewer cells were stained with anti-FcγR mAbs than with Leu-M5. Staining with anti-FcγRIII was weaker than staining with mAbs to FcγRI and FcγRII. Occasional perivascular cells were also stained by all three anti-FcγR mAbs. Control sections without primary mAbs were negative.

Phagocytosis

Eighty-eight per cent of purified human microglia (Fig 3A) formed rosettes with

EA (Fig 3B). Eighty-five percent of microglia ingested EA (Fig 3C). These numbers increased to 97% for rosetting and 96% for phagocytosis ($P < 0.0001$) after treatment with 100 U IFN-γ. The phagocytic index increased after IFN-γ-treatment, from 5.5 to 10.3 EA per microglia ($P = 0.01$). There was no rosetting or phagocytosis of EA-F(ab')$_2$ or E. Blocking of FcγR with immune complexes resulted in a reduction of rosetting microglia to 28%. No EA were ingested after FcγR-blocking. Immune complexes prepared of IgG F(ab')$_2$ fragments did not reduce rosetting or phagocytosis of EA.

ADCC

In the ADCC experiments, microglia lysed EA in a dose-dependent manner, but did not lyse EA-F(ab')$_2$ or E. As shown in Fig 4, IFN-γ increased microglial specific killing from 52% to 64% ($P=0.004$). Blocking of FcγR with immune complexes almost abolished the ADCC (3% specific killing), whereas immune complexes prepared of IgG F(ab')$_2$ fragments did not block ADCC.

Oxidative burst

Ninety-six per cent of microglia mounted an oxidative burst after PMA triggering. Although 85% of microglia ingested EA, only 48% gave an oxidative burst after EA ingestion. After IFN-γ treatment, 76% of microglia gave an oxidative burst after EA ingestion ($P=0.002$). Anti-FcγRI (37%), anti FcγRII (44%) and anti-FcγRIII (21%) mAbs all induced an oxidative burst. When the three mAbs were mixed together, 50% of the cells gave an oxidative response (Fig 3D). In control experiments, microglia were stimulated with E, EA-(F(ab')$_2$, or Leu-M5. Less than 7% of the cells gave an oxidative response.

OKT3 induced T cell mitogenesis

As shown in Table 2, microglia supported OKT3 induced T cell activation. After IFN-γ treatment of microglia, T cell activation was strongly enhanced (P=0.0008). Purified T cells were not stimulated by OKT3 mAb alone, thus verifying the absence of monocytes. Microglia alone did not stimulate allogeneic T cells to proliferate in a three day mitogen assay system.

DISCUSSION

In the present study we have assessed the expression of FcγR on human adult CNS cells in situ and in vitro, and the potential functional consequences of their presence on microglia, the cell type found to express FcγR in human CNS. In cell cultures from adult CNS, microglia were identified by Leu-M5 immunostaining. They were negative for Leu-M3 and non-specific esterase activity, distinguishing them from Leu-M5 positive cells isolated form peripheral blood (16). The microglia, like monocyte-derived macrophages (26) and alveolar and peritoneal macrophages (27), react with immune complexes and with mAbs to FcγRI, FcγRII and FcγRIII. FcγRIII on microglia was insensitive to PI-PLC treatment, as has been described for FcγRIII on macrophages (20). In contrast, FcγRIII on granulocytes is reported to be PI-linked (28).

Our in vitro immunohistochemical data are in accord with both our in situ studies of the surgically-derived CNS tissue and with previous data derived from autopsy tissue (5,6, Ulvestad et al., data to be published) which show that microglia in the parenchyma are immunostained with mAbs identifying all three classes of FcγR. Oligodendrocytes and astrocytes did not have FcγR. Fetal neurons up to 12 weeks gestational age are also FcγR negative (unpublished observation). The selective expression of FcγR on microglia only is further supported by the findings of Hayes et al. (29) who showed that microglia but not oligodendrocytes or astrocytes rosetted

with erythrocytes coated with IgG.

Previous immunohistochemical studies of active MS lesions have demonstrated capping of anti-myelin IgG on the surface of macrophages (8) and phagocytosed immune complexes within microglia (30), suggesting that FcγR-mediated phagocytosis plays a role in myelin breakdown. These observations are in accord with our functional in vitro data which show that exposure of human microglia to opsonized antigen (EA) results in ADCC and phagocytosis of the targets. Such data may indicate that blocking of FcγR mediated functions could abrogate myelin destruction in MS. Monocytes from patients treated with intravenous immunoglobulins have a decreased phagocytic activity due to FcγR modulation (31), and a similar mechanism could theoretically be operative in active MS lesions due to leakage of IgG across the damaged blood-brain-barrier (30). Our observation that microglia pretreated with immune complexes have a decreased phagocytic and cytotoxic activity in vitro, experimental data showing that systemic injection of serum IgG promotes CNS remyelination in mice (32), and a recent therapeutic trial indicating that intravenous immune globulins given to MS patients suppress the ongoing pathological process (33), are all in accordance with this hypothesis. In the normal CNS, FcγR on microglia likely function to clear immune complexes. In the inflamed CNS, IgG-FcγR interactions may acquire pathogenetic significance. Immune complexes crosslinking FcγR on monocytes and macrophages induce release of cytotoxic oxygen radicals (34). We have shown that also microglia can be triggered via all three FcγR to mount an oxidative burst. Oxygen radicals have been implicated in oligodendrocyte killing in vitro (11) and as mediators of vascular permeability and brain injury in vivo (12). Oligodendrocytes may thus be damaged as "innocent bystanders" rather than as antigen-restricted targets of the immune response. This hypothesis is further supported by data showing that crosslinking of FcγR on monocytes induces release of TNF (35) which is reported to be cytotoxic for oligodendrocytes in vitro (36).

Microglia are efficient accessory cells for antigen-specific T cell activation (16). In addition to presentation of antigen in the context of the MHC, accessory cells secrete T cell-activating soluble mediators. Crosslinking of FcγR on monocytes results in the production of IL-1 (37), IL-6 (38), and TNF (35), all implicated in T cell activation. To investigate whether microglia have the capability to produce T cell-stimulatory cytokines after FcγR-crosslinking, OKT3 mAbs were used in an anti-CD3-induced T cell proliferation assay. In this assay, CD3 complexes on the T cell surface are crosslinked via FcγR on the accessory cell. The following T cell proliferation is dependent upon soluble mediators produced by accessory cells and upon an IFN-γ-inducible physical interaction between accessory cells and T cells (39). Our results show that microglia are highly efficient T cell activators in this assay. The production of soluble mediator molecules might be of functional importance in inflammatory CNS diseases, and the FcγR dependent release points to a connection between immune complex generation and T cell activation.

The basis for the observed increase in disease activity in MS after systemic IFN-γ treatment remains speculative (13). IFN-γ administered in vivo augments expression of FcγR on human monocytes (40), and Vass et al. (10) recently demonstrated that IFN-γ potentiates antibody-dependent demyelination in rats in vivo. We have in the present study shown that FcγR-triggered responses such as phagocytosis, ADCC, oxidative burst and OKT3 induced T cell activation are all significantly enhanced after IFN-γ treatment of microglia in vitro. Such data may indicate that the deleterious effects of IFN-γ in MS could involve potentiation of demyelination via FcγR dependent functions.

The effects of IFN-γ on FcγR modulation has been studied in detail in monocytes and macrophages. Treatment of monocytes, monocyte-derived macrophages and peritoneal macrophages with IFN-γ results in increased numbers of FcγRI (41), determined by an IFN-γ responsive region on the gene encoding FcγRI (42). Since

the OKT3-induced mitogenesis assay is totally dependent on functional FcγRI activity (34), our data indicate that IFN-γ upregulates microglial FcγRI via similar mechanisms. Functional assays using EA as stimulators activate cells through all three FcγR. The effect of IFN-γ in these assays may be more complex, since IFN-γ also modulates FcγRII and FcγRIII expression and activity (43,44).

Since microglial cells in this study were obtained from epileptic patients undergoing surgery, we can not exclude the possibility that the microglia may be somewhat activated in vivo, neither can we exclude the possibility that isolation and culture conditions may activate the cells in vitro. The basal functional microglial activities we have observed may therefore not be representative for resting microglia in situ, but may instead be manifestations of the potential functions such cells may acquire when activated. Our data support the notion that microglia may, when interacting with opsonized antigen, cause functional responses which may be capable of inducing oligodendrocyte or myelin injury. FcγR on human microglia may consequently be of pathogenic significance in demyelinating diseases of the CNS.

REFERENCES

1. Fontana A, Fierz W, Wekerle H. Astrocytes present myelin basic protein to encephalitogenic T-cell lines. Nature 1984;307:273-76.

2. Boje KM, Arora PK. Microglial-produced nitric oxide and reactive nitrogen oxides mediate neuronal cell death. Brain Research 1992;587:250-56.

3. Matsumoto Y, Ohmori K, Fujiwara M. Immune regulation by brain cells in the central nervous system: microglia but not astrocytes present myelin basic protein to encephalitogenic T cells under in-vivo-mimicking conditions. Immunology 1992;76:209-16.

4. Perry VH, Hume DA, Gordon S. Immunohistochemical localization of macrophages and microglia in the adult and developing mouse brain. Neuroscience 1985;15:313-326.

5. Peress NS, Siegelman J, Fleit HB, Fanger MW, Perillo E. Monoclonal antibodies identify three IgG Fc receptors in normal human central nervous system. Clin Immunol Immunopathol 1989;53:268-80.

6. Akiyama H, McGeer PL. Brain microglia constitutively express β-2 integrins. J Neuroimmunol 1990;30:81-93.

7. Nyland H, Matre R, Mørk S. Fc receptors on microglial lipophages in multiple sclerosis. N Engl J Med 1980;302:120-21.

8. Prineas JW, Graham JS. Multiple sclerosis: Capping of surface immunoglobulin G

on macrophages engaged in myelin breakdown. Ann Neurol 1981;10:149-58.

9. Xiao BG, Linington C, Link H. Antibodies to myelin-oligodendrocyte glycoprotein in cerebrospinal fluid from patients with multiple sclerosis and controls. J Neuroimmunol 1991;31:91-96.

10.Vass K, Heininger K, Schäfer B, Linington C, Lassmann H. Interferon-γ potentiates antibody-mediated demyelination in vivo. Ann Neurol 1992;32:198-206.

11. Kim YS, Kim SU. Oligodendroglial cell death induced by oxygen radicals and its protection by catalase. J Neurosci Res 1991;29:100-06.

12. Chan PH, Schmidley JW, Fishman RA, Longar SM. Brain injury, edema, and vascular permeability changes induced by oxygen-derived free radicals. Neurology 1984;34:315-20.

13. Panitch HS, Hirsch RL, Schindler J, Johnson KP. Treatment of multiple sclerosis with gamma interferon: exacerbations associated with activation of the immune system. Neurology 1987;37:1097-1102.

14. Traugott U, Lebon P. 1988. Multiple sclerosis: involvement of interferons in lesion pathogenesis. Ann Neurol 1988;24:243-51.

15. Yong VW, Antel JP. Culture of glial cells from human brain biopsies. In: Richardson A, Fedoroff S, eds. Manual for nerve tissue culture. St. Louis U.S.A: The Humana Press Inc, 1992:81-96.

16. Williams K, Bar-Or A, Ulvestad E, Olivier A, Antel JP, Yong VW. Biology of adult human microglia in culture: comparisons with peripheral blood monocytes and astrocytes. J Neuropathol Exp Neurol 1992;51:538-49.

17. Pellegrino MA, Ferrone S, Theofilopoulos AN. Isolation of human T and B lymphocytes by rosette formation with 2-aminoethylisothiouronium bromide (AET)-treated sheep red blood cells and with monkey red blood cells. J Immunol Methods 1976;11:273-279.

18. Stanworth DR, Turner MW. Immunochemical analysis of human and rabbit immunoglobulins and their subunits. In Weir DM, ed. Handbook of experimental immunology, Vol. 1. Oxford UK:Blackwell Sci Publ, 1986:12.1-12.46.

19. Matre R, Haugen, Å. The placental Fcγ receptors studied using immune complexes of peroxidase. Scand J Immunol 1978;8:187-93.

20. Edberg JC, Barinsky M, Redecha PB, Salmon JE, Kimberly RP. FcγRIII expressed on cultured monocytes is a N-glycosylated transmembrane protein distinct from FcγRIII expressed on natural killer cells. J Immunol 1990;144:4729-34.

21. Yam LT, Li CY, Crosby WH. Cytochemical identification of monocytes and granulocytes. Am J Clin Pathol 1971;55:283-90.

22. Newman SL, Mikus LK, Tucci MA. Differential requirements for cellular cytoskeleton in human macrophage complement receptor and Fc receptor-mediated phagocytosis. J Immunol 1991;146:967-74.

23. Boot JHA, Geerts MEJ, Aarden LA. Functional polymorphisms of Fc receptors in human monocyte-mediated cytotoxicity towards erythrocytes induced by murine isotype switch variants. J Immunol 1989;142:1217-1223.

24. Badwey JA, Robinson JM, Karnovsky MJ, Karnovsky ML. Reduction and excitation of oxygen by phagocytic leucocytes: biochemical and cytochemical techniques. In Weir DM, ed. Handbook of experimental immunology, Vol. 1. Oxford UK: Blackwell Sci Publ, 1986:50.1-50.16.

25. Schwab R, Crow MK, Russo C, Weksler ME. Requirements for T cell activation by OKT3 monoclonal antibody: role of modulation of T3 molecules and interleukin 1. J Immunol 1985;135:1714-18.

26. Clarkson SB, Ory PA. CD16. Developmentally regulated IgG Fc receptors on cultured human monocytes. J Exp Med 1988 167:408-17.

27. Anderson CL, Looney RJ, Culp DJ et al. Alveolar and peritoneal macrophages bear three distinct classes of Fc receptors for IgG. J Immunol 1990;145:196-201.

28. Selvaraj P, Carpèn O, Hibbs ML, Springer TA. Natural killer cell and granulocyte Fcγ receptor III (CD16) differ in membrane anchor and signal transduction. J Immunol 1989 143:3283-88.

29.Hayes GM, Woodroofe MN, Cuzner ML. Characterisation of microglia isolated from adult human and rat brain. J Neuroimmunol 1988;19:177-89.

30. Gay D, Esiri M. Blood-brain barrier damage in acute

multiple sclerosis plaques. An immunocytological study. Brain 1991;114:557-72.

31. Kimberly RP, Salmon JE, Bussel JB, Crow MK, Hilgartner MW. Modulation of mononuclear phagocyte function by intravenous γ-globulin. J Immunol 1984;132:745-50.

32. Rodriguez M, Lennon VA. Immunoglobulins promote remyelination in the central nervous system. Ann Neurol 1990;27:12-17.

33. Achiron A, Pras E, Gilad R, et al. Open controlled therapeutic trial of intravenous immune globulin in relapsing-remitting multiple sclerosis. Arch Neurol 1992;49:1233-36.

34. Van de Winkel JGJ, Anderson CL. Biology of human immunoglobulin G Fc receptors. J Leukoc Biol 1991;49:511-24.

35. Debets JMH, van de Winkel JGJ, Ceuppens JL, Dieteren IEM, Buurman WA. Cross-linking of both FcγRI and FcγRII induces secretion of tumor necrosis factor by human monocytes, requiring high affinity Fc-FcγR interactions. J Immunol 1990;144:1304-10.

36. Selmaj KW, Raine CS. Tumor necrosis factor mediates myelin and oligodendrocyte damage in vitro. Ann Neurol 1988;23:339-46.

37. Remvig L, Thomsen BS, Baek L, Svenson M, Bendtzen K. Interleukin 1, but not interleukin 1 inhibitor, is released from human monocytes by immune complexes. Scand J Immunol 1990;32:255-61.

38. Krutmann J, Kirnbauer R, Köck A et al. Cross-linking Fc receptors on monocytes triggers IL-6 production. Role in anti-CD3-induced T cell activation. J Immunol 1990;145:1337-42.

39. Kawakami K, Yamamoto Y, Kakimoto K, Onoue K. Requirement for delivery of signals by physical interaction and soluble factors from accessory cells in the induction of receptor-mediated T cell proliferation. Effectiveness of IFN-γ modulation of accessory cells for physical interaction with T cells. J Immunol 1989;142:1818-25.

40. Maluish AE, Urba WJ, Longo DL et al. The determination of an immunologically active dose of interferon-gamma in patients with melanoma. J Clin Oncol 1988;6:434-45.

41.Guyre PM, Girard MT, Morganelli PM, Manganiello PD. Glucocorticoid effects on the production and actions of immune cytokines. J Steroid Biochem 1988;30:89-93.

42. Pearse RN, Feinman R, Ravetch JV. Characterization of the promoter of the human gene encoding the high-affinity IgG receptor: Transcriptional induction by γ-interferon is mediated through common DNA response elements. Proc Natl Acad Sci USA 1991;88:11305-09

43. Van Schie RCAA, Verstraten HGG, Tax WJM, van den Berkmortel FWPJ, van de Winkel JGJ, de Mulder PHM. Effect of rIFN-γ on antibody-mediated cytotoxicity via human monocyte IgG Fc receptor II (CD32). Scand J Immunol 1992;36:385-93.

44. Andreesen R, Brugger W, Scheibenbogen C, Kreutz M, Leser HG, Rehm A, Löhr GW. Surface phenotype analysis of human monocyte to macrophage maturation.

J Leukoc Biol 1990;47:490-97.

Table 1. Antibodies used for immunostaining.

Antigen	Antibody	Cell specificity	Dilution	Source[a)]
CD3	OKT3	T cells	1:1000	Ortho
CD11c	Leu-M5	Monocyte/macrophage	1:10	B/D
CD14	Leu-M3	Monocyte/macrophage	1:10	B/D
CD16/FcγRIII	3G8	Leukocyte	1:10	Medarex
CD32/FcγRII	IV.3	Leukocyte/platelet	1:50	Medarex
CD59	1F5	Widely distributed	1:250	N. Okada
CD64/FcγRI	32.2	Monocyte/macrophage	1:20	Medarex
GalC[b)]	H8H9	Oligodendrocytes	1:1	S. Kim
GFAP[c)]	GFAP	Astrocytes	1:100	Dakopatts

a) Ortho: Ortho Pharmaceutical Corporation, Raritan, NJ. B/D: Becton Dickinson, Mountain View, Cal. Medarex: Medarex Inc., West-Lebanon, NH. N. Okada, Fukuoka, Japan. S. Kim, Vancouver, Canada. Dakopatts: Dakopatts, Glostrup, Denmark. b) GalC: Galactocerebrocide. c) GFAP: Glial fibrillary acidic protein.

Table 2. OKT3-mediated activation of T cells.

Culture conditions	[^{3}H]Thymidine incorporation
MG + OKT3	336 ± 243
MG + OKT3 + IFN-γ	130 ± 40
T + OKT3	141 ± 18
MG + T	165 ± 50
MG + T + OKT3	20,735 ± 915
MG + T + OKT3 + IFN-γ	34,858 ± 2.503

1000 microglia (MG) or 1000 microglia co-cultivated with 10^5 allogeneic T cells (T) in the presence or absence of OKT3 mAb (2.5 ng/ml) and IFN-γ (100 U/ml).

FIGURE LEGENDS

Figure 1. Double immunofluorescence to detect FcγR on human cultured microglia. AB, CD, and EF are of the same fields. A: FcγRI, C: FcγRII, E: FcγRIII. B, D, and F: GFAP. (x760).

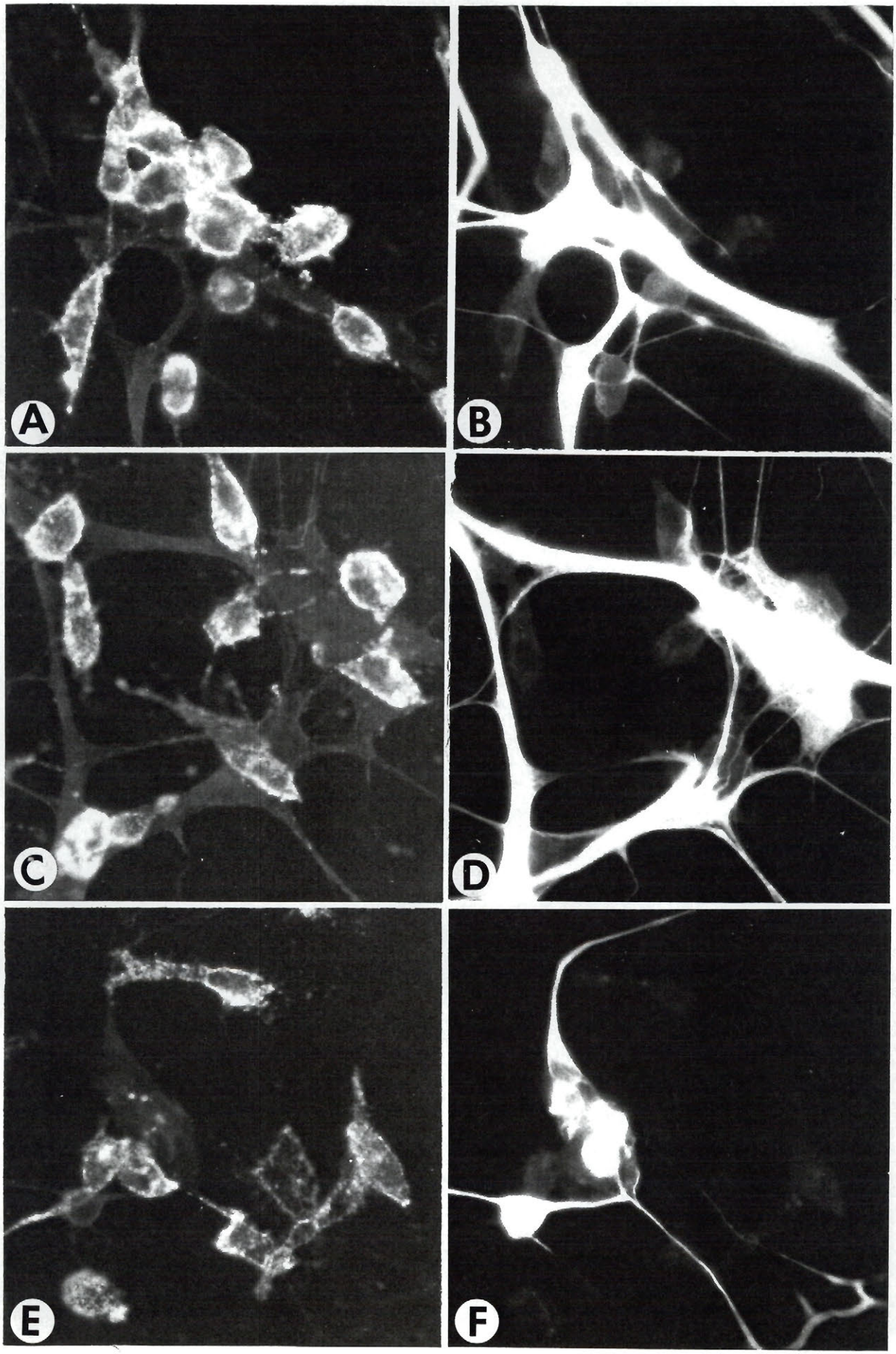
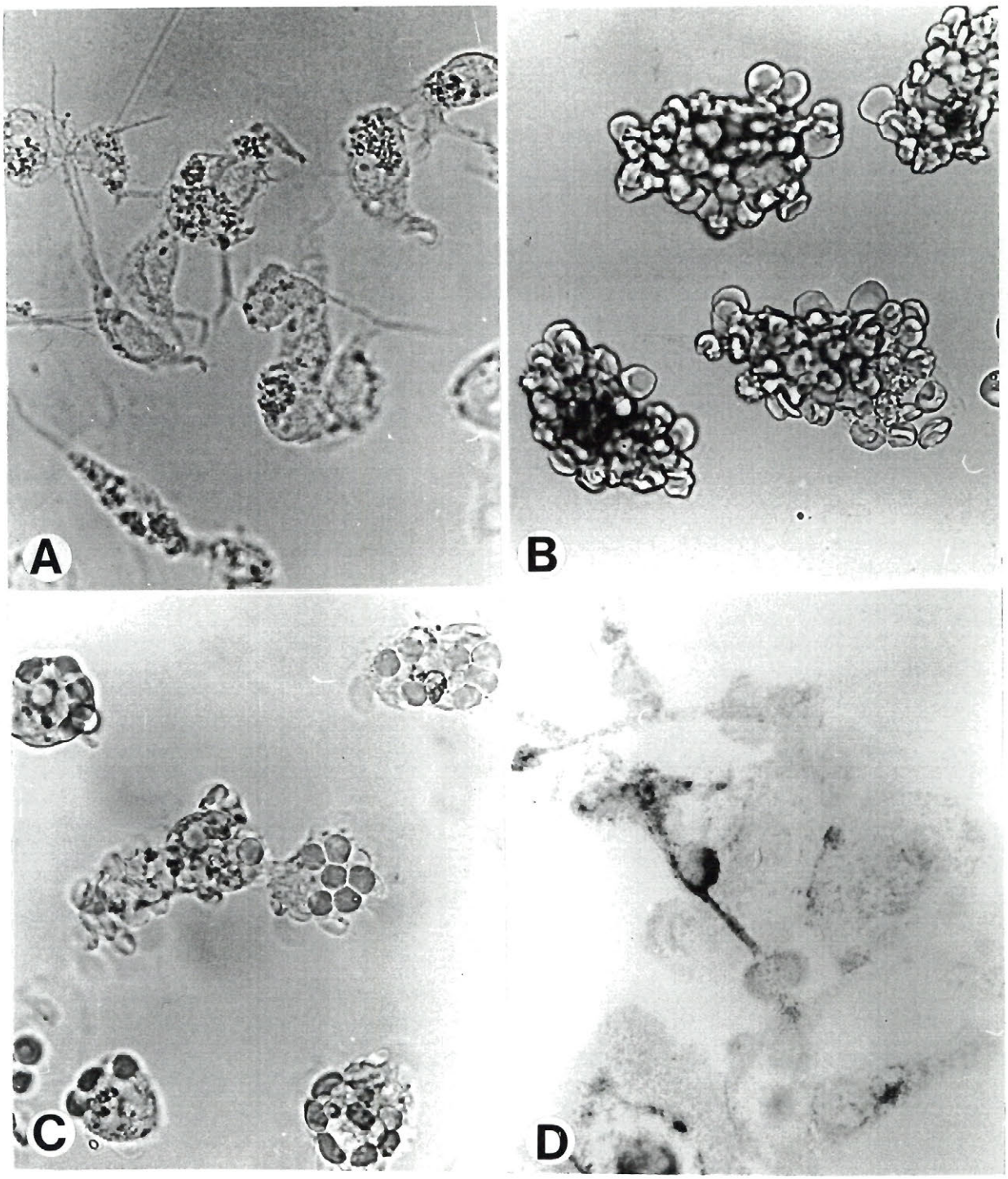

A
B
C
D
E
F

Figure 2. Immunoperoxidase staining of cryostat sections from a tissue block of surgically resected adult CNS tissue. Counterstaining with hematoxylin. A: Leu-M5, B: FcγRI, C: FcγRII, and D: FcγRIII. (x375).

A
B
C
D

Figure 3. Enriched seven day microglial cultures. A: Untreated microglia. B: Binding of opsonized erythrocytes (EA) to microglial FcγR. C: Phagocytosed EA within microglia. D: Reduced nitroblue tetrazolium within microglia treated with mAbs to FcγRI, FcγRII and FcγRIII. (x760).


A
B
C
D

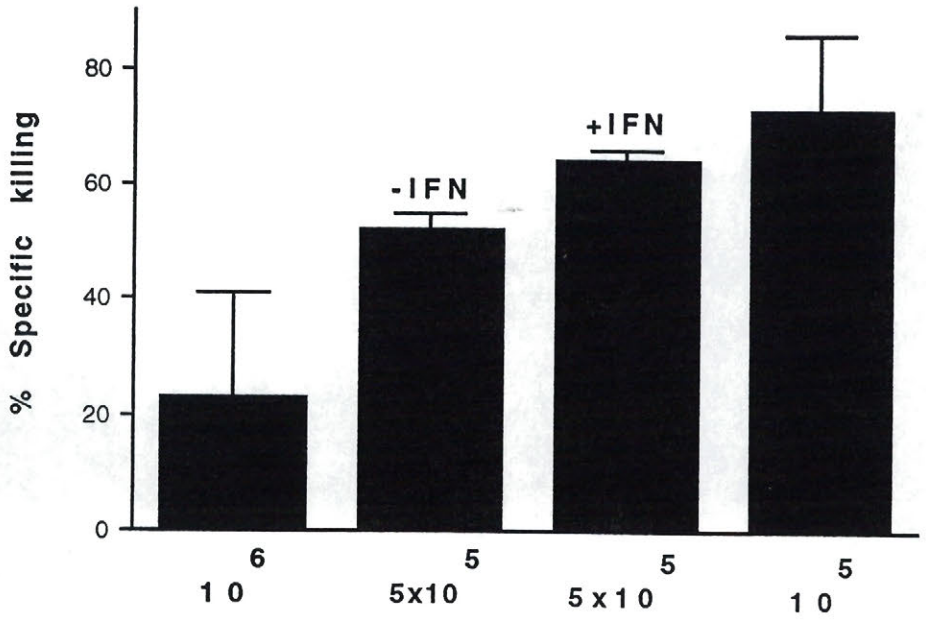


Fig. 4 ADCC activity of human microglia cultured at 10^5 cells per well. EA added at three different cell numbers, 10^6, 5 x 10^5 and 10^5. Microglia challenged with 5 x 10^5 EA were pretreated either with or without IFN-γ. Data are given as % specific killing $\pm$ SD. Results are expressed as [^{3}H]-thymidine cpm $\pm$ SD (n=3).

Chapter 8

REACTIVE MICROGLIA IN MULTIPLE SCLEROSIS LESIONS HAVE AN INCREASED EXPRESSION OF RECEPTORS FOR THE Fc PART OF IgG

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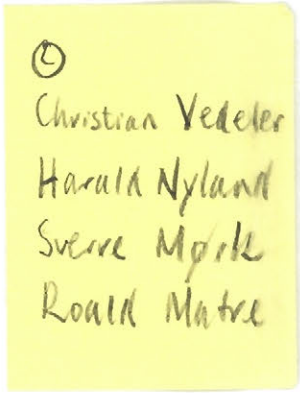
Jack Antel, Harald Nyland, Sverre Mørk, and Roald Matre

Preface

Data presented in this chapter, follow upon the demonstration of FcR expression on microglia in vitro and in situ from "normal" tissue specimens, and the observation that IFN gamma resulted in increased microglial FcR dependent T cell activation in the OKT3 assay (Chapter 7). Data presented in this chapter are the result of the investigation of FcR expression on microglia in MS brain materials. Major points demonstrated in this chapter include:

1. Confirmation of results presented in Chapter 7 that normal human white matter have low level of expression of FcR I-III.

2. The demonstration of upregulated FcR expression in active MS lesions over that of non-lesion areas.



ABSTRACT

Receptors for the Fc part of IgG, FcRI (CD64), FcRII (CD32), and FcRIII (CD16) were studied by indirect immunoperoxidase staining of cryostat sections from normal and multiple sclerosis (MS) brains. Microglia in the parenchyma of normal white matter had a dendritic morphology, and were weakly stained by monoclonal antibodies (mAbs) to FcRI, FcRII, and FcRIII. In active MS lesions reactive microglia were strongly stained by the mAbs 32.2 (FcRI), IV.3 (FcRII), and 3G8 (FcRIII). Perivascular macrophages were stained by all anti-FcR mAbs in both normal white matter and in MS lesions, whereas endothelial cells were stained by the anti-FcRIII mAb only. The FcR on microglia and perivascular macrophages may be of functional importance in antibody-dependent cell-mediated cytotoxicity (ADCC), phagocytosis, and local immunoregulation. FcR on endothelium may be of importance in binding and transportation of immune complexes into the CNS. FcR mediated functions may consequently be highly relevant to the pathogenesis of MS.

INTRODUCTION

Multiple sclerosis (MS) is a chronic disease in the human central nervous system (CNS) white matter of unknown etiology and pathogenesis, characterized pathologically by multiple foci of demyelination with varying degrees of perivascular inflammation and gliosis. The identification in MS lesions of blood vessel damage and blood-brain barrier leakage suggests that endothelial dysfunction is of importance in the pathogenesis of MS (1,2). Perivascular lymphocytic aggregates and increased local CNS production of oligoclonal immunoglobulins, some of which are reactive with myelin, indicate involvement of the cellular and humoral parts of the immune system in the demyelinating process (3,4).

Important links between the cellular and humoral branches of the immune system are provided by FcR, making the study of these molecules essential for our understanding of immunologic regulation and immune reactivity in health and disease. The availability of monoclonal antibodies (mAbs) against FcR has contrib-

uted substantially to identification and functional characterization of these molecules, and three classes of FcR have been identified on human leukocytes on the basis of ligand affinity, reactivity with mAbs, and cloning of complementary DNA (cDNA) (5). FcRI (CD64) is a 72 kDa molecule with high affinity for IgG, expressed on monocytes and macrophages. FcRII (CD32) has a molecular weight of 40 kDa and is found on monocytes, macrophages, neutrophils, B cells, and platelets. FcRIII (CD16) with a molecular weight between 50 and 70 kDa, is expressed on neutrophils, NK cells, and macrophages. FcRII and FcRIII have low affinity for IgG.

The functional importance of FcR in the human CNS is still not fully characterized, but data indicate that microglia and perivascular macrophages both have the capability to phagocytose myelin debris, apparently with IgG as ligand between myelin and FcR on the phagocytosing cell (6-8). FcR positive cells may however also play an active part in the demyelinating process. The binding to FcR of myelin-specific IgG may induce antibody-dependent cell-mediated cytotoxicity (ADCC) and release of cytotoxic mediators by microglia and perivascular macrophages (9), effects which may lead to killing of oligodendrocytes and consequently demyelination.

Such potentially deleterious consequences of FcR triggering indicate that FcR may be of pathogenetic significance in MS. To further explore the role of FcR in CNS disease we have therefore studied the expression of FcR in normal white matter and in MS lesions using immunohistochemical methods. In vivo observations were correlated with in vitro results from human microglia maintained in tissue culture.

MATERIALS AND METHODS

Patients

Six patients with clinically definitive MS were studied (Table 1). The patients had

suffered from MS between three and fifteen years, and the diagnosis was confirmed by post mortem examination of the CNS. Three patients with non-cerebral diseases were included as controls.

Tissue

Brain and spinal cord from MS patients and controls were obtained at various time intervals after death. Blocks of tissue from MS lesions and surrounding white matter as well as white matter from the controls were frozen in liquid nitrogen and sectioned (4-6 μm) on a cryostat. Histological staining of the sections with Luxol fast blue was performed to delineate demyelinated areas, with hematoxylin and eosin to reveal mononuclear cells within the lesions, and with Oil red O to identify cells with neutral fat in their cytoplasm. Hypercellular lesions with perivascular inflammatory cells and numerous Oil red O positive cells were assessed as active.

Microglia cultures

Isolated glial cells were obtained from patients undergoing surgical procedures for intractable epilepsy by a previously described protocol (10). Microglia were cultivated for three days before analysis in Eagle's minimum essential medium supplemented with 5% FCS, gentamicin 20 μg/ml, glucose 1 mg/ml, and 2 Mm glutamine, from GIBCO, Burlington, Ont.

Antibodies

The mAbs used in this study are summarized in Table 2. MAbs 32.2 and 3G8 were used as $F(ab')_2$ fragments and mAb IV.3 as Fab fragments. Rabbit anti-human von Willebrand factor, biotinylated rabbit anti-mouse immunoglobulins, and horseradish peroxidase (HRP)-conjugated swine antibodies to rabbit IgG were purchased from Dakopatts, Glostrup, Denmark. Rhodamine-conjugated goat anti-mouse immunoglobulins and fluorescein-conjugated goat anti-rabbit immunoglobulins were obtained from Cappel, West Chester, PA. Immune complexes of

HRP and rabbit IgG anti-HRP or rabbit F(ab')$_2$ fragments of IgG anti-HRP were prepared as described (11).

Immunohistochemistry

Sections were fixed in cold acetone for 5 min. They were thereafter incubated with 10% rabbit serum diluted in phosphate buffered saline, pH 7.2 (PBS) for 30 min at room temperature, followed by over night incubation at 4^{0}C in a humid chamber with mAbs diluted in 10% rabbit serum. Biotinylated rabbit anti-mouse immunoglobulins diluted 1:300 were then applied for 30 min. Avidin-biotin-peroxidase complex (ABComplex/HRP, Dakopatts code K 355) was prepared as recommended by the manufacturer, and allowed to react with sections for 30 min. Sections were finally treated with a 3-amino-9-ethyl-carbazole containing buffer for the development of a colored reaction product. All incubations were followed by washings in PBS.

Tissue sections incubated with immune complexes over night at 4^{o}C in a humid chamber were incubated with HRP-conjugated swine anti-rabbit IgG diluted 1:100 for 30 min, and developed as described above. All sections were counterstained with hematoxylin, mounted in Glycergel (Dakopatts), and analyzed by light microscopy.

Immunofluorescence analysis

Tissue sections were fixed in cold acetone for 5 min and then incubated with immune complexes or anti-human von Willebrand factor over night in a humid chamber. Sections were thereafter incubated with fluorescein-conjugated goat anti-rabbit antibodies for 45 min. Leu-M5 or 3G8 were thereafter introduced for 2 hours, followed by rhodamine-conjugated goat anti-mouse immunoglobulins for 45 min. Sections were finally mounted in Gelvatol (Air Products and Chemicals Inc, Allentown, PA).

Cells on cover slips were analyzed by indirect immunofluorescence labelling

techniques as previously described (12,13). All incubations were performed at 4°C and followed by washings in PBS. Controls consisted of experiments in which the primary antibodies were omitted. A Leitz microscope equipped with rhodamine and fluorescein optics was used for immunofluorescence analysis.

Demonstration of non-specific esterase (NSE)

The cytochemical reaction for NSE was performed according to the method described by Yam et al.(14).

RESULTS

Normal white matter

Perivascular cells but not parenchymal cells were stained by NSE and Leu-M3. Leu-M5 and KP1 reacted with perivascular cells and with ramified cells throughout the parenchyma. All anti-FcR mAbs stained ramified cells in the parenchyma. Anti-FcRI and anti-FcRII mAbs gave a stronger reactivity than the anti-FcRIII mAb. FcRI, FcRII, and FcRIII were demonstrated on infrequent perivascular cells. Some endothelial cells were stained by the anti-FcRIII mAb, but not by anti-FcRI or anti-FcRII mAbs. Immune complexes reacted with endothelium and perivascular cells, but also with a few scattered cells in the parenchyma.

Active MS lesions

Diffuse cellular NSE reactivity was found on cells in the perivascular area (Fig 1A). A few scattered cells in the parenchyma were also NSE positive. A similar staining was observed with Leu-M3. Leu-M5 (Fig 1B) and KP1 (Fig 1C) reactivity was prominent on perivascular cells and on cells throughout the lesions. The parenchymal cells within the lesions and at the lesion edge had an oval morphology with Few if any ramified processes, typical for reactive microglia. Cells in the surrounding parenchyma had a more ramified morphology. The staining with KP1 was cytoplasmic and granular, in contrast to the more membranous staining

observed with Leu-M5.

The mAb 32.2 against FcRI showed a strong reaction with reactive microglia throughout the lesions (Fig 1D), the immunostaining pattern was similar to the staining with the Leu-M5 mAb in serial sections. A similar reaction was observed with the anti-FcRII mAb IV.3 (Fig 1E). The anti-FcRIII mAb 3G8 (Fig 1F) gave a weaker staining than did 32.2 and IV.3. The staining intensity of all three mAbs was strongest on reactive microglia within the lesions and at the lesion border (Fig 2A). Microglia in the normal appearing parenchyma surrounding the lesions had a more ramified morphology and exhibited staining characteristics comparable to microglia in control brains. The reactivity with anti-FcR mAbs was evenly distributed on the cell membranes, and no polar staining was observed. All mAbs to FcR reacted with perivascular cells (Fig 2B). The anti-FcRIII mAb 3G8 stained elongated cells in small venules (Fig 2C), identified as endothelial cells in double fluorescence with antibodies against von Willebrand factor. Endothelium was not stained by mAbs against FcRI and FcRII.

Soluble immune complexes (HRP-anti-HRP) bound to endothelium and to cells both in the perivascular spaces and in the parenchyma (Fig 2D, Fig 2E). Perivascular and parenchymal cells reactive with immune complexes were also Leu-M5 positive as identified by double immunofluorescence staining. $F(ab')_2$ fragments of immune complexes did not react.

Cultured microglia

Microglia in culture were stained by Leu-M5 and KP1, but were negative for NSE and Leu-M3. They were also stained by mAbs reactive with FcRI, FcRII and FcRIII. More than 85% of cultured Leu-M5 positive microglia bound HRP-anti-HRP immune complexes (Fig 2F). When analyzed in a fluorescence microscope, cultured microglia had multiple small autofluorescent granula in their cytoplasm (Fig 2F). A similar autofluorescence was observed within reactive microglia in MS lesions (Fig 2E).

DISCUSSION

In the present paper we have demonstrated binding of immune complexes and anti-FcR mAbs to cells in the parenchyma, to perivascular cells, and to endothelium in normal and diseased human CNS (Table 3). FcR expression was highly upregulated on perivascular and parenchymal cells within active MS lesions as compared to FcR expression on cells in the parenchyma located outside the demyelinating lesion. This was evident from the enhanced binding of anti-FcR mAbs and immune complexes to cells within MS lesions. Various inflammatory mediators with capability to induce expression and functional activity of FcR on phagocytic cells, such as interferon-γ and tumor necrosis factor (9,15), have been identified in MS lesions (16,17). The increased FcR expression on microglia in active lesions may consequently reflect microglial activation by such mediators. No specific microglial marker is as yet available, and in an attempt to discriminate between resident microglia and invading monocytes we therefore utilized enzymatic staining with NSE and the mAb Leu-M3, both recognizing macrophages and monocytes but not microglia (13,18). NSE and Leu-M3 reactivity was found almost exclusively on perivascular infiltrating cells. These <u>in situ</u> data were further confirmed by our <u>in vitro</u> studies, establishing that microglia are Leu-M5/KP1/FcR positive but NSE/Leu-M3 negative. FcR on perivascular cells and microglia probably mediate functions such as binding of immune complexes, phagocytosis of opsonized particles, ADCC, and release of inflammatory mediators (9,18). FcR-mediated functions may consequently be of pathogenetic significance in demyelination. Of particular interest is the fact that FcR-mediated uptake of antigens strongly enhance antigen presentation by myeloid cells (19), thereby providing a link between T and B cells with similar antigenic specificity.

We have previously demonstrated FcRIII on endothelial cells of human placenta and peripheral nerves (20,21), and now report FcRIII on endothelial cells of small venules in the CNS. FcRIII mediates phagocytosis of immune complexes in macrophages and neutrophils (9). The functional significance of endothelial FcRIII

is however poorly defined. Since FcRIII has low affinity for monomeric IgG but binds strongly to immune complexes, a possible function of FcRIII on endothelium might be binding and transportation of immune complexes into the CNS, making the blood-brain barrier permeable for antigens that have already provoked an antibody response in the peripheral immune system. Perivascular macrophages and probably also microglia may thereafter phagocytose the transported immune complexes, leading to antigen degradation and presentation of peptide fragments in the HLA class II molecules of these cells (19). Since antigen-activated T cells transgress the intact blood-brain barrier in what appears to be a random fashion, a continuous immunologic surveillance of the CNS is thereby accomplished, preparing the CNS for a specific immune attack against potential deleterious invaders (20,21). An immune response would according to this hypothesis be initiated around small venules in the CNS, thus explaining the vasoconcentricity of T cells in MS and viral encephalitis. Such a hypothesis could also explain why antigens present in the CNS that have elicited an immune response in the periphery vigorously induce a CNS immune response, whereas antigens present in the CNS and to which the peripheral immune system has not been exposed, are tolerated (22). It is well established that FcR on myeloid cells are important effectory and regulatory molecules. Further functional in vitro experiments are however needed to establish the role of FcR on microglia and on endothelial cells, and to verify or refute the hypotheses proposed in this paper.

REFERENCES

1. Kermode, A.G., Tofts, P.S., MacManus, D.G., Kendall, B.E., Kingsley, D.P.E., Moseley, I.F., du Boulay, E.P.G.H. and McDonald, W.I. (1988) Early lesions of multiple sclerosis. Lancet, ii: 1203-1204.

2. Gay, D. and Esiri, M. (1991) Blood-brain barrier damage in acute multiple sclerosis plaques. An immunocytological study. Brain., 114: 557-572.

3. Nyland, H., Mørk, S. and Matre, R. (1982) In-situ characterization of mononuclear cell infiltrates in lesions of multiple sclerosis. Neuropathol. Appl. Neurol., 8: 403-411.

4. Tourtellotte, W.W., Walsh, M.J., Baumhefner, R.W., Staugaitis, S.M. and Shapshak, P. (1984) The current status of multiple sclerosis intra-blood-brain-barrier IgG synthesis. In: Scheinberg, L.C. and Raine, C.S. (Eds.), Multiple sclerosis: experimental and clinical aspects Ann. NY Acad. Sci., 436: 52-67.

5. Ravetch, J.V. and Anderson, C.L. (1990) FcγR family: Proteins, transcripts, and genes. In: Metzger, H (Ed.), Fc receptors and the action of antibodies. American Society for Microbiology, Washington. 211-235.

6. Nyland, H., Matre, R. and Mørk, S. (1980) Fc receptors on microglial lipophages in multiple sclerosis. N. Engl. J. Med., 302: 120-121.

7. Prineas, J.W. and Graham, J.S. (1981) Multiple sclerosis: Capping of surface immunoglobulin G on macrophages engaged in myelin breakdown. Ann. Neurol., 10: 149-158.

8. Peress, N.S., Siegelman, J., Fleit, H.B., Fanger, M.W. and Perillo, E. (1989)

Monoclonal antibodies identify three IgG Fc receptors in normal human central nervous system. Clin. Immunol. Immunopathol., 53: 268-280.

9. Van de Winkel, J.G.J. and Anderson, C.L. (1991) Biology of human immunoglobulin G Fc receptors. J. Leukoc. Biol.,49: 511-524.

10. Yong, V.W. and Antel, J.P. (1992) Culture of glial cells from human brain biopsies. In: Richardson, A. and Fedoroff, S. (Eds.), Protocols for neural cell culture. The Humana Press Inc., St. Louis, U.S.A. 81-96

11. Matre, R. and Haugen, A. (1978) The placental Fcγ receptors studied using immune complexes of peroxidase. Scand. J. Immunol., 8: 187-193.

12. Williams, K, Ulvestad E, Blain, M, J.P. Antel. Induction of primary T cell responses by human glial cells. J Neurosci Res 36: (In Press).

13. Williams, K., Bar-Or, A., Ulvestad, E., Olivier, A., Antel, J.P. and Yong, V.W. (1992) Biology of adult human microglia in culture: comparisons with peripheral blood monocytes and astrocytes. J. Neuropathol. Exp. Neurol., 51: 538-549.

14. Yam, L.T., Li, C.Y. and Crosby, W.H. (1971) Cytochemical identification of monocytes and granulocytes. Am. J. Clin. Pathol., 55: 283-290.

15. Loughlin, A.J., Woodroofe, M.N. and Cuzner, M.L. (1992) Regulation of Fc receptor and major histocompatibility complex antigen expression on isolated rat microglia by tumour necrosis factor, interleukin-1 and lipopolysaccharide: effects on interferon-gamma induced activation. Immunology, 75: 170-175.

16. Traugott, U. and Lebon, P. (1988). Multiple sclerosis: Involvement of interferons in lesion pathogenesis. Ann. Neurol., 24: 243-251.

17. Selmaj, K., Raine, C.S., Cannella, B. and Brosnan, C.F.(1991) Identification of lymphotoxin and tumor necrosis factor in multiple sclerosis lesions. J. Clin. Invest., 87: 949-954.

18. Hayes, G.M., Woodroofe, M.N. and Cuzner, M.L. (1988) Characterization of microglia isolated from adult human and rat brain. J. Neuroimmunol., 19: 177-189.

19. Gosselin, E.J., Wardwell, K., Gosselin, D.R., Alter, N.,Fisher, J.L. and Guyre, P.M. (1992) Enhanced antigen presentation using human Fcγ receptor (monocyte/macrophage)-specific immunogens. J. Immunol., 149: 3477-3481.

20. Kristoffersen, E.K., Ulvestad, E., Vedeler, C.A. and Matre, R. (1990) Fcγ receptor heterogeneity in the human placenta. Scand. J. Immunol., 31: 561-564.

21. Vedeler, C.A., Matre, R., Kristoffersen, E.K. and Ulvestad, E. (1991) IgG Fc receptor heterogeneity in human peripheral nerves. Acta Neurol. Scand., 84: 177-180.

22. Hickey, W.F. and Kimura, H. (1988) Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vivo. Science, 239: 290-292.

23. Hickey, W.F. (1991) Migration of hematogenous cells through the blood-brain barrier and the initiation of CNS inflammation. Brain Pathol., 1: 97-105.24.

24. Mason, D.W., Charlton, H.M., Jones, A.J., Lavy, C.B.D., Puklavec, M. and

Simmonds, S.J. (1986) The fate of allogeneic and xenogeneic neuronal tissue transplanted into the third ventricle of rodents. Neuroscience, 19: 685-694.

TABLE 1

SUMMARY OF MULTIPLE SCLEROSIS CASES STUDIED

Case No.	Autopsy	Age	Sex	Death - post mortem interval (hours)	Number of lesions studied
1	O.091/86	52	F	8	2
2	O.095/86	37	F	37	2
3	O.570/86	41	F	8	3
4	O.756/91	56	F	24	3
5	R.106/92	42	F	41	3
6	O.614/92	43	M	45	4

TABLE 2

MONOCLONAL ANTIBODIES USED FOR IMMUNOHISTOCHEMICAL ANALYSIS

Antigen	Antibody	Cell specificity	Dilution	Source
CD11c	Leu-M5	Monocyte/Macrophage	1:10	B/D
CD14	Leu-M3	Monocyte/Macrophage	1:10	B/D
CD16/FcRIII	3G8	Leukocyte	1:10	Medarex
CD32/FcRII	IV.3	Leukocyte/Platelet	1:50	Medarex
CD64/FcRI	32.2	Monocyte/Macrophage	1:20	Medarex
CD68	KP1	Monocyte/Macrophage	1:50	Dakopatts

B/D: Becton Dickinson, Mountain View, Cal., USA.

Medarex: Medarex Inc., West-Lebanon, NH, USA.

Dakopatts: Dakopatts, Glostrup, Denmark.

TABLE 3

BINDING OF ANTI-FcR ANTIBODIES TO ENDOTHELIAL, PERIVASCULAR AND PARENCHYMAL CELLS IN NORMAL WHITE MATTER AND IN ACTIVE MS LESIONS

		FcRI	FcRII	FcRIII
Normal CNS	Endothelial cells	-	-	+
	Perivascular cells	+	+	+
	Parenchymal cells	+	+	+
Active lesion	Endothelial cells	-	-	+
	Perivascular cells	++	++	++
	Parenchymal cells	++	++	++

- negative, + positive, ++ strongly positive

FIGURE LEGENDS

Figure 1. A: Non specific esterase staining of perivascular cells in MS lesion. B: Leu-M5 (CD11c) on reactive microglia in MS lesion. C: CD68 (KP1) on perivascular cells and on microglia in MS lesion. D: FcRI on reactive microglia in MS lesion. E: FcRII on reactive microglia in MS lesion. F: FcRIII on reactive microglia in MS lesion. B - F: Immunostaining, ABComplex/HRP. Counterstaining with hematoxylin. x200.

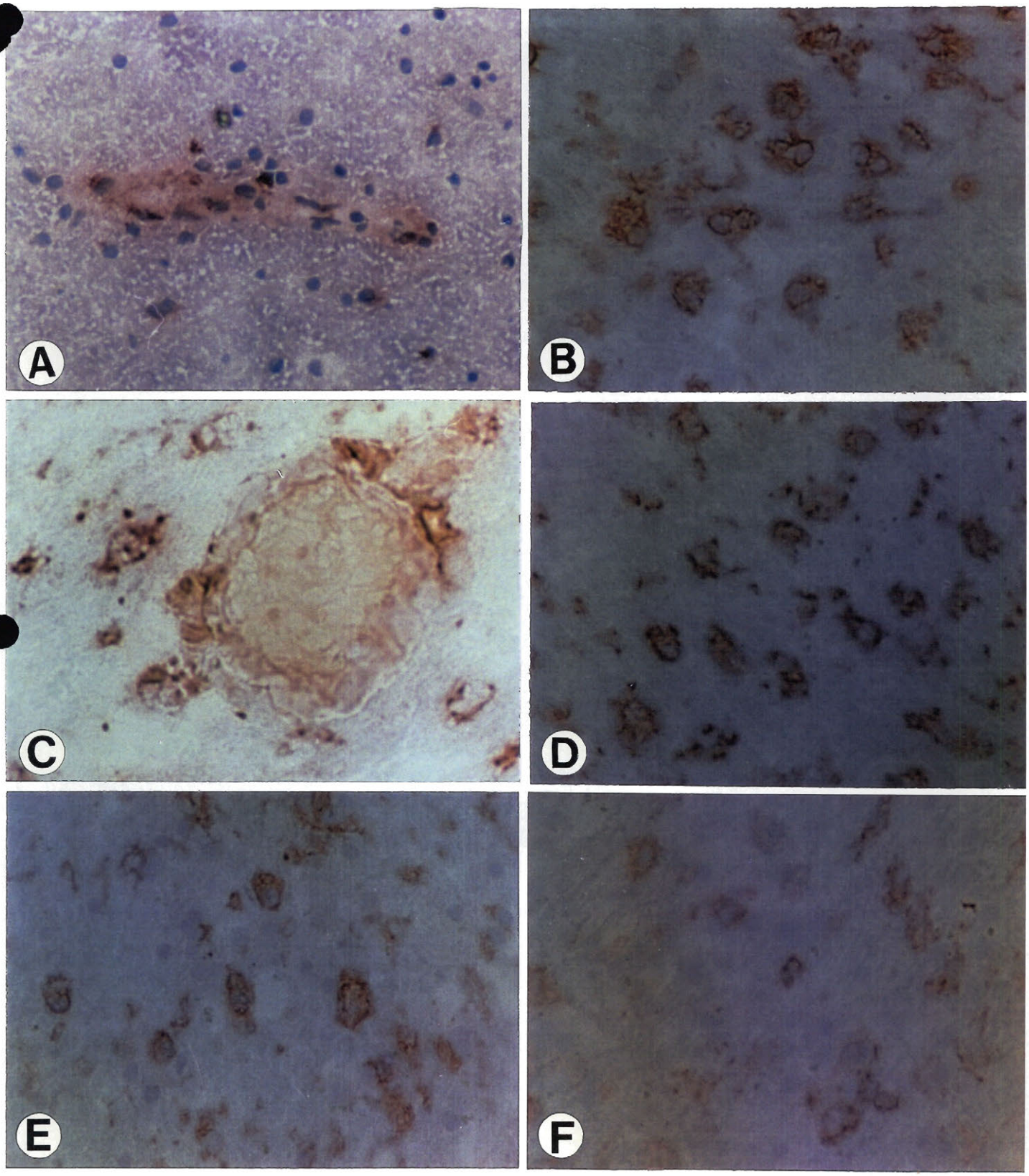
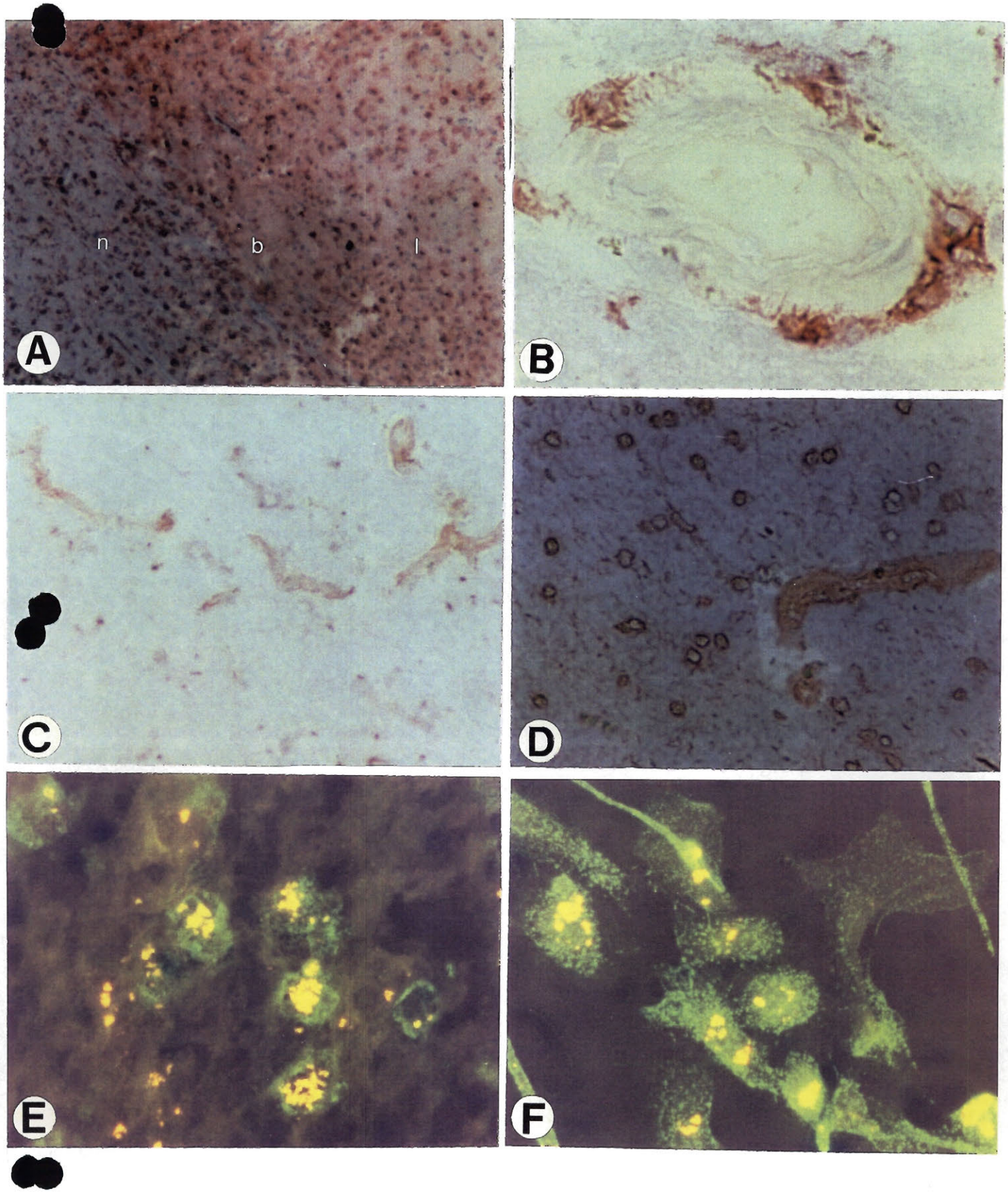

A
B
C
D
E
F

Figure 2. A: FcRI on reactive microglia at the border of an active MS lesion. l=lesion, b=border zone, n=normal parenchyma. x40. B: FcRII on perivascular cells in MS brain. x200. C: FcRIII on microglia and endothelial cells (arrow) in MS brain outside the demyelinating focus. x200. D: Immune complex (HRP-anti-HRP) binding to endothelium (open arrow) and reactive microglia (small arrow) in MS lesion. x100. E: Immune complex (HRP-anti-HRP) binding to reactive microglia in MS lesion. x400. F: Immune complex (HRP-anti-HRP) binding to cultured microglia. x630. A - D: Immunostaining, ABComplex/HRP. Counter-staining with hematoxylin. E - F: FITC-staining.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Chapter 9

ACTIVATION OF ADULT HUMAN DERIVED MICROGLIA BY MYELIN PHAGOCYTOSIS IN VITRO

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Preface

Chapters 7 and 8 demonstrated the expression of FcR I-III on microglia in vitro and corresponding upregulation of microglial FcR expression in active lesions of multiple sclerosis brain material. Following the observation that the opsonization of antigen can facilitate uptake of antigen and resultant antigen presentation by presenting cells, and from the observation of myelin debris in macrophages and microglia in multiple sclerosis lesions, it was sought to determine the capacity of microglia to phagocytose myelin. Comparisons were made between uptake of myelin that was not treated and myelin that had been opsonized with an anti-myelin mAb and human serum. The consequence of myelin phagocytosis by microglia was investigated in terms of microglial activation using an oxidative burst and cytokine assays. Major points presented in this chapter include:

1. The demonstration that microglia can phagocytose myelin vesicles in vitro where the level of myelin uptake by microglia increases following the opsonization of myelin vesicles.

2. Myelin phagocytosis by microglia results in the activation of microglia seen by the increased number of cells undergoing oxidative burst and by increased cytokine production.

ABSTRACT

The present study was designed to determine the extent to which cultured human glial cells phagocytose normal CNS myelin and CNS myelin opsonised with serum or purified antibody against MBP. Glial cells studied were mixed cultures (consisting of astrocytes, microglia, and oligodendrocytes) and enriched microglia cultures established from adult human brain specimens, and enriched astrocytes from fetal human brain. A human monocytic cell line, THP-1, was included as a control. Uptake of ^{125}I-labelled myelin was followed over a 24 hr time period. An assay of oxidative burst (30 min) and cytokine bioassays measuring IL-1, IL-6, and TNF (6-48 hr) production were used to investigate short and long term activation of phagocytosing cells. Maximum myelin uptake by glial cells occurred within 12 hr following myelin incubation. Opsonization of myelin prior to the phagocytosis assay resulted in greater myelin uptake by mixed glial cell cultures, microglia, and THP-1 cells over that of non-treated myelin. The magnitude of myelin phagocytosis by astrocytes was considerably lower than microglia and THP-1, and was not affected by myelin opsonization. Within 30 minutes of myelin phagocytosis, microglia and THP-1 cells underwent oxidative burst; opsonization of myelin by purified anti-MBP IgG and heat-inactivated serum enhanced the microglia oxidative burst activity. Production of IL-1, TNF and most markedly IL-6 by microglia was increased following 12 to 24 hr of myelin ingestion. Our data demonstrate that myelin phagocytosis by adult human microglia occurs in vitro, and is augmented when myelin is opsonized. Myelin phagocytosis by microglia results in the activation of microglia as assessed by oxidative burst and cytokine production.

INTRODUCTION

Multiple sclerosis (MS), a putative central nervous system (CNS) autoimmune disease, is characterized by multifocal areas of perivascular inflammation, gliosis, and demyelination. The precise bases for the tissue injury in MS remain to be defined. Microglia and macrophages within active MS lesions exhibit an activated phenotype including up-regulated MHC class II antigens (1,2) and Fc receptor (FcR) expression (2-4). Activated microglia and macrophages have been implicated as mediators of oligodendrocyte and/or myelin injury via cell-cell contact dependent mechanisms, including antibody-dependent cell mediated cytotoxicity (ADCC) (5-7). Microglia/macrophage activation can be induced by cytokines produced by activated T cells, and by immunoglobulin (IgG) binding to Fc receptors on microglia (3). Intrathecal production of IgG, some of which are OL/myelin directed, is an almost universal occurrence in MS.

Previous studies have shown that rodent derived macrophages (8,9) and microglia (10) actively phagocytose myelin in vitro and that magnitude of myelin uptake, measured by the levels of cholesterol ester production, could be increased following the opsonization of myelin with whole serum, serum gamma globulin, or purified anti-myelin Abs (9,10) . It has also been shown that the titres of IgG from the cerebrospinal fluid (CSF) of rabbits with experimental allergic encephalomyelitis (EAE) correlated with the severity of disease (9). We have previously reported a protocol for the isolation and culture of adult human derived microglia and have shown that in vitro, we can distinguish between microglia (Leu-M5$^+$, Leu-M3$^-$ and non-specific esterase (NSE) negative) and autologous peripheral blood derived monocyte/macrophages (Leu-M5$^+$, Leu-M3$^+$, and NSE$^+$) (3,11). Additionally, we have shown that adult human derived microglia express FcRs I-III in vitro (3,11) and in vivo (4), and that microglia can bind, phagocytose, and lyse opsonized target cells via FcRs (3).

In the current study, we investigated the ability of adult human derived microglia, fetal human astrocytes, and a human monocyte cell line- THP-1, to phagocytose myelin vesicles in vitro; assessing the effects of myelin opsonization on facilitating myelin

uptake in a 24 hr assay. We further investigated the possibility of microglial activation as a consequence of myelin phagocytosis over a 30 minute time period; measuring oxidative burst responses using the reduction of nitroblue tetrazolium, and over a 24 hour time period, using cytokine bioassays to monitor the production of IL-1, IL-6, and TNF.

MATERIALS AND METHODS

Source of glial and monocytic cells - Adult human brain tissue served as the source of mixed glial and enriched microglia cultures and was obtained from surgical resections, carried out to ameliorate non-tumor related intractable epilepsy (N=5 patients) (ages 20-45). Tissues used were from regions requiring resection to reach the precise epileptic focus, and were distant from the main electrically active site. Fetal human CNS tissue were the source of astrocytes and were prepared from 8-12 week old specimens (n=4) following Medical Research Council of Canada approved guidelines. The THP-1 monocytic cell line was obtained from ATCC, TIB202.

Glial cell preparation

Preparation of mixed glial cultures and enriched microglia cultures -

Our method of preparing mixed glial cell cultures and cultures of enriched microglia from adult human CNS materials has been previously described (11,12). Briefly, tissue was treated with 0.25% trypsin in the presence of DNase (50 μg/ml) followed by a percoll gradient centrifugation (Pharmacia LKB Biotechnology, Uppsala, Sweden) at 15,000 RPM for 30 min. The resulting dissociated cell suspension comprised of mixed glial cells (microglia, astrocytes, oligodendrocytes) was then suspended in culture medium consisting of Eagle's minimal essential medium (MEM) supplemented with 5% fetal calf serum (FCS), 0.1% glucose, and 20 μg/ml gentamicin, and seeded directly into 24 well plates for phagocytosis assays as described below, or into 25 cm^2 tissue culture flasks (Nunc, Burlington, Ontario). The following day, floating cells

consisting mainly of oligodendrocytes, were removed. Remaining cells consisting of microglia and astrocytes were allowed to develop morphologically for 7 days at which time the less adherent astrocytes were then floated off by rotary shaking for 5 hr at 150 rpm. Microglia were detached by incubation of cells with 0.25% trypsin and DNase (50 μg/ml) at 37°C for 15 min, and seeded into 24 well plates at densities described below. Mixed glial cell preparations and enriched microglia cultures were also seeded onto Aclar fluorocarbon coated coverslips (Dr. S. Kim, Vancouver, British Columbia) previously coated with 10 μg/ml poly-L-lysine. Mixed glial cell cultures from various adult human donors consisted of 40-70% microglia (Leu-M5$^+$), 20 - 50% astrocytes (GFAP$^+$), and 15 - 25 % oligodendrocytes (GalC$^+$). Enriched microglia cultures (>95% Leu-M5$^+$) (11) were Leu-M3$^-$ and NSE$^-$, thus distinguishing them from autologously derived peripheral blood monocyte/macrophages (Leu-M3$^+$, NSE$^+$) (3,11). Adult human derived astrocytes cannot be derived in sufficient numbers or enriched beyond 70% purity (13) and therefore could not be used in this study which required more highly enriched cultures.

Preparation of astrocyte cultures -

Fetal cultures were prepared by carefully stripping CNS material of meninges and blood vessels, mechanically dissociating the tissue with scalpel blades followed by treatment with trypsin (0.25%) and DNase (50μg/ml) at 37 degrees C for 45 min. Dissociated tissue was then passed through a 130 μm nylon mesh, washed 2X in phosphate buffered saline (PBS) and cells were plated directly onto poly-L-lysine coated plastic culture dishes in culture medium (13). Cultures consisting of astrocytes, neurons, and sparse microglia, were split every 2 weeks when confluent. Following the second or third passage, when fetal neurons and microglia were no longer apparent, astrocytes were removed by treatment with trypsin (0.05%) and seeded into 24 well plates for experiments described below. Astrocyte cultures were established in excess of 95% purity as seen by immunofluoresence with antibodies against GFAP (14).

Myelin vesicle preparation and iodination -

Myelin isolated from normal human brain white matter was suspended in 2mM phosphate buffer pH 8.0 at a final concentration of 0.2 mg/ml, and incubated overnight at 4°C. Myelin vesicles were pelleted by centrifugation at 15,000 RPM for 30 min and the pellet resuspended in 5 mM phosphate buffer pH 8.4 at a final concentration of 5 mg/ml. After vortexing, the vesicle solution was passed through a 25 gauge needle and the resulting vesicles stored at 4°C and used within 48 hr (15). Myelin vesicles were iodinated by the method of Markwell (16) using Iodobeads (Pierce, Illinois, USA). Briefly, 100 μg of myelin vesicles were incubated with 500 μCi $Na^{125}I$, in the presence of Iodobeads for 15 min at 37°C. The specific activity of the myelin was variable and ranged between 8,700 to 50,000 cpm/μg total myelin protein.

Phagocytosis assay

For these studies mixed glial cell cultures, enriched microglia, enriched astrocyte cultures, and THP-1 cells were seeded into 24 well plates at $1X10^4$ cells per well (microglia, astrocytes, THP-1) and $4X10^4$ cells per well (mixed glial cultures). The THP-1 cells were initially cultured in RPMI medium with 5% FCS prior to phagocytosis assays. In order to make the THP-1 cells adherent for the phagocytosis assays, they were cultured in serum free medium and stimulated with PMA (100 nM) for 24 hr. ^{125}I-Myelin vesicles were opsonized using either a purified anti-MBP mAb (IgG2a, against myelin peptide 130-137, Boehringer Mannheim, Burlington, Ontario, Canada), or heat-inactivated human serum (Pel Frez, Dearborn, WI) (56°C, 30 min, to deactivate complement). For opsonization, myelin vesicles were incubated with serum or anti-MBP mAb for 1 hr or overnight at 37°C at a concentration of 100 μg IgG of anti-MBP mAb or 100μl of human serum per 100 μg of myelin protein. Following myelin opsonization, myelin vesicles were washed extensively with PBS. Myelin was added to cultures at a final concentration of 10 μg/ml for 0 to 24 hr. At designated time points (0hr, 6hr, 12hr, 24hr) conditioned medium from phagocytosis assays was removed, centrifuged at 12,000 rpm for 10 min, and snap frozen in liquid nitrogen for cytokine bioassays. At

the end of the phagocytosis assays, cells were washed 3X with PBS before harvesting by lysing the cells with distilled water, 1 ml per well. Radioactive counts of triplicate wells were determined using a gamma counter (Fischer, Laval, Quebec) and expressed as mean cpm $\pm$sem. Myelin uptake was expressed as the quotient of the total radioactive counts of triplicate cultures over the cpm per μg of myelin protein. Determination of the non-specific binding of myelin to cells was done by incubating cells at 4°C to block phagocytosis.

Confocal Imaging of Phagocytosis

Myelin basic protein was incubated with FITC-Celite (Sigma, St. Louis, MO) in 0.1M $NaHC0_3$ pH 9.0 for 1 hr at 25 degrees C. Myelin basic protein (MBP) was washed in PBS to remove celite beads and free uncoupled FITC. FITC-labelled MBP was incubated with microglia cells ($1X10^4$ cells per coverslip) for 30 minutes at either 37°C or at 4°C. Coverslips were washed twice in PBS, fixed lightly in 2% PFA and mounted onto slides. Confocal images were generated on a Leica Laser Tecknik Gmb H microscope (Leica, Heidelberg, Germany) by an argon laser with a line of 488 nM. Emitted fluorescence light was detected through a band pass filter peak at 535 $\pm$ 7 nM before transmission to the photomultiplier. The image was reconstructed from the average of 16 passes per raster line.

Oxidative burst

Nitroblue tetrazolium (NBT) acts as a non-specific electron acceptor in biochemical pathways activated during the oxidative burst, producing a blue precipitate within the reactive cell. NBT (Sigma, St Louis, MO) was dissolved in 0.9% NaCl at a final concentration of 1 mg/ml. PMA (Sigma) a potent protein kinase C activator, was used at a final concentration of 10 nM or 100 nM. Enriched microglia and astrocytes ($2 X 10^4$ cells) seeded on coverslips were incubated with 30 μl NBT and either 20 μl PMA, or myelin vesicles. After 45 min, cells were fixed in 4% glutaraldehyde and mounted. Coverslips were examined using a light microscope, and results expressed as

RESULTS

Myelin phagocytosis

Figure 1 depicts the results of the phagocytosis of opsonized and non-opsonized myelin vesicles by mixed glial cell cultures, the activated monocytic cell line THP-1, and enriched microglia and astrocyte cultures. Mixed glial cell cultures (4 X 10^4 cells per well), consisting of microglia (40-70%) astrocytes (20-30%), and oligodendrocytes (15-25%), reached a maximal myelin uptake at 12 hr of incubation (4μg). The uptake of myelin opsonized with heat- inactivated serum was higher than non-opsonized myelin at 12 hours (2μg) (Figure 1A).

The activated monocytic cell line, THP-1, had the largest mean uptake of myelin which peaked at 24 hr (Figure 1B). There was no marked difference between opsonized myelin versus non-opsonized myelin by THP-1 cells within the first 12 hr of phagocytosis. Phagocytosis by THP-1 cells after 24 hr was highest for myelin that had been opsonized with anti-MBP mAb (50μg) followed by the uptake of myelin opsonized with heat-inactivated serum (22μg) and non-treated vesicles (9μg).

Maximal myelin uptake by enriched microglia cultures was seen at 12 hr where vesicles that had been opsonized with heat-inactivated serum were taken up most efficiently (7μg) (Figure 1C). There was no detectable difference in phagocytosis by microglia of vesicles that had been opsonized with purified anti-MBP mAb (4μg) or non-opsonized myelin (4μg). Enriched astrocyte cultures had a low level of myelin uptake (0.5μg) compared to the uptake of mixed glial cell cultures or enriched microglia (Figure 1D).

Phase-contrast microscopic analysis of live cultures demonstrated the accumulation of myelin vesicles by microglia within 6 hr of incubation, but not by enriched astrocyte cultures (0-24 hr) (data not shown). Confocal microscopy was used to distinguish phagocytosis of myelin vesicles from fluid phase pinocytosis and cell surface binding. Confocal microscopy studies using fluorescein labelled-MBP demonstrate microglia phagocytosis of MBP (Figure 2A). The uptake of MBP by

microglia could be completely blocked by the incubation of microglia at 4°C, an indicator of phagocytosis rather than pinocytosis (Figure 2B). Similar results were obtained for THP-1 cells.

Oxidative burst

To define early effects of myelin phagocytosis by glial and THP-1 cells, oxidative burst was measured by the reduction of NBT in a 30 min assay. The results of the NBT assay can been seen in individual cells where a blue, insoluble precipitate forms (Figure 3). Table 1 demonstrates that following incubation with 10 nM PMA, 80% of enriched microglia cultures and 88% of the THP-1 cells undergo oxidative burst. Only 4% of the astrocytes were positive for the reaction product using 10 nM PMA. Following incubation with 100 nM PMA, 41% of the astrocytes were positive for the NBT reduction product. The phagocytosis of untreated myelin by microglia resulted in 7% of the cells undergoing oxidative burst. The number of microglia undergoing oxidative burst increased to 21% using myelin vesicles incubated with the anti-MBP mAb, and 69% using vesicles opsonized with heat-inactivated serum. Enriched astrocyte cultures did not undergo significant oxidative burst in response to any of the above stimuli. The THP-1 cells showed a 22% oxidative burst with non-treated vesicles. The percent of THP-1 cells positive for the NBT reaction product increased to 37% with anti-MBP mAb, and to 35% when vesicles were opsonized with heat-inactivated serum.

Cytokine bioassays

Cytokine bioassays of conditioned medium from the phagocytosis assays demonstrate a concordant increase in the production of IL-1, IL-6, and TNF by microglia over time correlating with the progressive increase in non-opsonized myelin uptake. Figure 4 represents results from an experiment demonstrating the kinetics of cytokine production by enriched microglial cultures in response to myelin phagocytosis. Of the three cytokines assayed, IL-6 levels were the highest with peak values of 9000 pg/ml at 48 hr; TNF peaked at 24 hr at 2,220 pg/ml; and IL-1 peaked at 48 hr at 501 pg/ml.

Table 2 presents the mean peak production of IL-1, IL-6, and TNF by mixed glial cells in comparison to enriched astrocyte and microglia cultures. Production of IL-6 (719 pg/ml) and TNF (213 pg/ml) by mixed glial cell cultures was lower than IL-1 and IL-6 produced by enriched microglial cultures, but higher than astrocytes (IL-6, 194 pg/ml; TNF, 161 pg/ml) following myelin uptake (Table 2). IL-1 production by astrocytes was not detected. The production of IL-6 and TNF by mixed glial cell cultures most likely represents the combination of the microglia and the astrocyte contribution.

DISCUSSION

In MS and EAE, the evidence for microglia and macrophage involvement in lesion formation include EM studies demonstrating phagocytosis of myelin at the edge of active lesions (21,22) and evidence of macrophages actively stripping myelin lamellae (23). Nyland et al., (24) first reported that microglia lipophages in MS lesions expressed Fc receptors. That myelin phagocytosis could involve Ig bound to myelin was further supported by studies demonstrating capping of IgG with myelin fragments in MS lesions (25) and the demonstration of myelin phagocytosis by clathrin-coated pits (22,26,27). Gay and Esiri (27) demonstrated immune complexes within microglia in MS pathology further supporting a role for microglia FcR in myelin phagocytosis. In accordance with these observations, we have recently shown that FcR I-III are upregulated on microglia within active MS lesions compared to their expression in non-lesion areas or in CNS tissues from non-inflammatory cases (3,4).

Elevated concentrations of oligoclonal IgG in the CSF are a hall mark of MS (28). The damaged blood brain barrier in MS, occuring during the course of the disease (27,29) may also allow serum and CSF derived IgG into the CNS parenchyma. Evidence suggests that some of the Abs are specific for oligodendrocytes and myelin (30). It has also been shown that normal, non-specific serum IgG can bind to myelin, resulting in opsonization of the myelin and phagocytosis (8). Antibodies found in MS and EAE CSF and serum can contribute to demyelination (31,32); one mechanism for such activity would be via macrophages or microglia serving as the effector cell.

The phagocytosis of myelin by rodent macrophages (8,9,33,34,35) and microglia (10,36) has been studied previously. Goldenberg et al., (8) observed increased uptake of myelin that had been treated either with anti-myelin antibodies or non-specific IgG in a short-term assay (120 min). Sommer et al., (8) demonstrated an increased percent of cholesterol esterification by rodent macrophages as an indicator of myelin phagocytosis following opsonization of myelin in a 30 hour assay.

Our data indicate that mixed human glial cells, comprising of microglia, astrocytes, and oligodendrocytes, can also phagocytose myelin vesicles in vitro. Purified

microglia and the monocyte cell line THP-1, but not fetal astrocytes, had high levels of myelin uptake that increased over time, and with myelin opsonization. Activated THP-1 cells had the highest level of myelin uptake after 12 hr. In order to have the THP-1 cells remain adherent it was necessary to treat the cells with PMA to activate them, thereby making them macrophage-like. Comparing the myelin uptake by microglia to that of THP-1, it is interesting to note that the microglia showed greater phagocytosis after opsonization with the human AB serum than with the anti-myelin mAb (IgG2A). The reverse is true with the THP-1 cells. One explanation for this may be that the IgG2a mAbs bind exclusively to FcRI (37). Since microglia are relatively freshly derived from the CNS, they may have cytophilic monomeric IgG bound to their FcRI (37,38), thereby blocking some interaction of FcRI with IgG2a mAbs. THP-1 cells have not been grown in serum containing IgG, thereby allowing their FcRI to freely interact with IgG2a mAbs. Furthermore, downregulation of microglial FcRI expression cannot be excluded. Observations indicate that monocytes downregulate FcRI expression when cultured in vitro (39). All three FcR have the capacity to mediate phagocytosis (40). The enhanced phagocytosis of myelin opsonized with human serum as compared to myelin opsonized with anti-myelin mAb by microglia, indicated that FcRII and FcRIII may play an important part in microglial phagocytosis. We have previously shown that human microglia phagocytose erythrocytes opsonized with IgG, but not unopsonized erythrocytes (3). It is therefore of interest to note the highly efficient phagocytosis of unopsonized myelin by human microglia. This may be the result of non-specific binding of myelin to scavenger receptors on microglia. Scavenger receptors have a high lipid binding capacity (41,42). Myelin consists of 70% lipid and 30% protein (43) and therefore the composition of lipids should be considered in defining mechanisms of phagocytosis. An increase in negatively charged lipids including phosphatidylcholine, phosphatidylserine, and phosphatidic acid in liposomes has been shown to increase cell surface binding to, and ultimately phagocytosis by, macrophages (44). This mechanism has been proposed to function through a scavenger receptor and may partially explain the enhanced uptake of myelin rather than RBC's by their respective membrane composition.

Results of the oxidative burst assay show that microglia, similar to THP-1 cells undergo oxidative burst in response to 10 nM PMA. Incubation of both cells with untreated myelin resulted in few cells undergoing oxidative burst. The number of microglia positive for the NBT reaction product increased when microglia phagocytosed myelin opsonized with anti-MBP mAb, and heat-inactivated serum. The increased respiratory burst activity seen by microglia using opsonized vesicles indicate a role for FcR crosslinking. We have previously demonstrated a similar increase in oxidative burst using opsonized red blood cells or purified antibody against individual FcR (3). Non-opsonized RBC's, similar to non-opsonized myelin, did not enhance oxidative burst activity. Oxygen radicals have been implicated in oligodendrocyte killing in vitro (45) and as mediators of vascular permeability and brain injury in vivo (46).

In terms of cytokine production, microglia had low basal levels of IL-1, IL-6, and TNF, just detectable at the beginning of the assay; following incubation with non-opsonized myelin vesicles, cytokine levels increased indicating activation of the cells. IL-1 was detected until 48 hr into the assay, IL-6 levels continued to increase with time in the assay peaking at 48 hr. TNF peaked at 24 hours. Non-myelin treated cultures did not demonstrate an increase in cytokine production over all time points investigated. IL-1, IL-6, and TNF production could be induced in our assay in response to non-specific phagocytosis of myelin. Previous studies have shown that crosslinking of FcR may induce the production of these cytokines (47,48). We have previously demonstrated, using an OKT3 T cell mitogenesis assay which is dependent on both functional FcR and cytokine production by the stimulating cell, that microglia can support the proliferation of purified $CD4^+$ T cells, thus implicating the production of cytokines as a result of FcR ligation by OKT3 mAbs. Human fetal astrocytes, which do not express any currently described FcR (3) had a low level of IL-6 and TNF production following incubation of myelin vesicles. Similar to others (49,50) we did not detect IL-1 production by astrocytes under basal culture conditions or following myelin incubation.

Each of the cytokines assayed in this study have been implicated in the development of MS lesions or heightened CNS immune reactivity. TNF has been

identified in MS brain (51) and has been implicated as a mediator of oligodendrocyte toxicity in vitro (52,53). IL-1 has been demonstrated to induce proliferation of adult astrocytes (54) and be involved in the production and expression of an array of cytokine mRNAs by fetal astrocytes (54,55), and might be important in promoting the proliferation of naive $CD4^+$ T cells in the CNS (Williams et al., submitted) thereby contributing to the ongoing intrathecal immune response characteristic of MS. IL-6 is known to stimulate B-cells resulting in the production of immune globulins and might therefore complete a feedback loop with microglia whereby cross-linking of FcR could lead to activation of microglia resulting in the production of IL-6 and continued immune globulin synthesis.

The current study has utilized adult human glial cell cultures. We demonstrated that myelin phagocytosis is mediated largely by microglia cells. Our data indicate that opsonization of myelin results in both increased myelin uptake and activation of microglia as seen in the oxidative burst assay. Furthermore, phagocytosis by adult human derived microglia results in the production of the cytokines IL-1, IL-6, and TNF; cytokine levels correlate with the extent of myelin accumulation. These data may be particularly interesting in the context of myelin injury within the CNS, where in contrast to the PNS, myelin degradation is slow sometimes occurring over the course of years (56). The slow removal of myelin in CNS inflammatory disease by microglia and macrophages phagocytosing myelin could result in chronic microglial activation with resultant reactive oxygen species generation and cytokine production.

REFERENCES

1. Boyle E.A. and McGeer P.L. (1990): Cellular Immune response in Multiply Sclerosis plaques. Am J Pathol 137:575-584.

2. McGeer PL, Kawamata T, Walker DG, Akiyama H, Tooyamam I., McGeer EG (1993): Microglia in Degenerative Neurological Disease. Glia 7:84-92.

3. Ulvestad E, Williams K, Matre R, Nyland H, Olivier A, Antel JP (1993a): Fc receptors for IgG on cultured microglia mediate cytotoxicity and phagocytosis of antibody-coated targets. J Neuropath Exp Neurol (In Press).

4. Ulvestad E, Williams K, Vedeler C, Antel JP, Nyland H, Mork S, Matre R (1993b): Reactive microglia in multiple sclerosis lesion have an increased expression of receptors for the Fc part of IgG. J Neurol Sci (In Press).

5. Brosnan CF, Stoner GL, Bloom BR, Wisniewski HM (1977): Studies on demyelination by activated lymphocytes in the rabbit eye: II antibody dependent cell-mediated demyelination. J Immunol 118:2103-2110.

6. Scolding NJ, Compston DAS (1991): Oligodendrocyte-macrophage interactions in vitro triggered by specific antibodies. Immunol. 72:127-132.8.

7. Zajkek JP, Wing M, Scolding NJ, Compston DAS (1992): Interactions between oligodendrocytes and microglia; a major role for complement and tumor necrosis factor in oligodendrocyte adherence and killing. Brain 115:1611-1631.

8. Goldenberg PZ, Kwoon EE, Benjamins JA, Whitaker JN, Quarles RH, Prineas JW (1989):Opsonization of normal myelin by anti-myelin antibodies and normal serum. J

Neuroimmunol 23:157-166.

9. Sommer MA, Forno LS, Smith ME (1992): EAE cerebrospinal fluid augments in vitro phagocytosis and metabolism of CNS myelin by macrophages. J Neurosci Res. 32:384-394.

10. Smith ME. (1993): Phagocytosis of myelin by microglia in vitro. J Neurosci Res 35:480-482.

11. Williams K, Bar-Or A, Ulvestad E, Olivier A, Antel JP, Yong VW (1992): Biology of adult human microglia in culture: comparisons with peripheral blood monocytes and astrocytes. J Neuropath Exp Neurol. 51:538-549.

12. Yong VW, Antel JP (1992): Culture of glial cells from human brain biopsies. In: Richardson A and Fedoroff,S. (Eds.). Protocols for neural cell culture. The Humana Press Inc. St. Louis, USA. 81-96.

13. Yong VW, Moumdjian R, Yong FP, Ruijs TCG, Freedman MS, Cashman N, Antel JP (1991): gamma-Interferon promotes proliferation of adult human astrocytes in vitro and reactive gliosis in the adult mouse brain in vivo. Proc Natl Acad Sci USA 88:7016-7020.

14 Williams KC, Dooley NP, Ulvestad E, Waage A, Blain M, Yong VW, Antel JP (1993): Antigen presentation by fetal human astrocytes: the cooperative effect of microglia or the microglial cytokine IL-1. (Submtted).

15. Steck AJ, Sirguist P, Zaller P, Herschkowiz NW, Schaefer R (1978): Preparation of membrane vesicles from isolated myelin. Studies on functional and structural properties. Biochem. Biophys. Acta 509:397-409.

16. Markwell MAK (1982): A new solid-state reagent to Iodinate proteins. Conditions for the efficient labelling of antiserum. Anal Biochem. 125:427-432.

17. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL.(1986): Two types of murine helper T cell clones. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 136:2348-2357.

18. Aarden LA, De Groot ER, Schaap OL, Lansdorp PM (1987): Production of hybridoma growth factor by human monocytes. Eur J Immunol 17: 1411-1416.

19. Espevik T, Nissen-Meyer J. (1986): A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. J Immunol Meth. 95:99-105.

20. Mosmann T. (1983): Rapid colorimetric assay for cellular growth and suvival: Application to proliferation and cytotoxicity assays. J Immunol Meth. 65:55-63.

21. Prineas JW (1975): Pathology of the early lesion in Multiply Sclerosis. Hum Pathol 6:531-554.

22. Prineas JW, Connell F (1978): The fine structure of chronically active multiple sclerosis plaques. Neurology. 28:68-75.

23. Lampert P, Carpenter S (1965): Electron microscopic studies on the vascular permeability and the mechanism of demyelination in experimental allergic encephalomyelitis. J Neuropath Exp Neurol. 24:11-24.

24. Nyland H, Matre R, Mork S (1980): Fc receptors on microglia lipophages in multiple sclerosis. N Engl J Med. 302:120-121.

25. Prineas JW, Graham JS (1980): Multiple sclerosis: capping of surface immunoglobulin G on macrophages engaged in myelin breakdown. Ann Neurol 10:149-158.

26. Epstein LG, Prineas JW, Raine CS (1983): Attachment of myelin to coated pits on macrophages in experimental allergic encephalomyelitis. J Neurol Sci. 61:341-348.

27. Gay D, Esiri M (1991): Blood-brain barrier damage in acute multiple sclerosis plaques. Brain 114:557-572.

28. Tourtellotte WW, Walsh MJ (1984): Cerebrospinal fluid profile in multiple sclerosis. In: CM Posner, DW Paty, L Scheinberg, WI McDonals, and GC Ebers (Eds.). The diagnosis of multiple sclerosis. Thieme-Stratton, New York, 165-178.

29. Esiri MM, Reading MC (1987): Macrophage populations associated with multiple sclerosis plaques. Neuropath and Applied Neurobiol 13:451-465.

30. Xiao BG, Linnington C, Link H (1991): Antibodies to myelin-oligodendrocyte glycoprotein in cerebrospinal fluid from patients with multiple sclerosis and controls. J Neuroimmunol. 31:91-96.

31. Linington C, Lassmann H (1987): Antibody response in clinical relapsing experimental allergic encephalomyelitis: correlation of serum demyelinating activity with antibody titre to myelin/oligodendrocyte glycoprotein (MOG). J Neuroimmunol. 17:61-69.

32. Linington C, Bradl M, Lassmann H, Brunner C, Vass K (1988): Augmentation of demyelination in rat acute allergic encephalomyelitis by circulating mouse monoclonal antibodies directed against a myelin/oligodendrocyte glycprotein. Am J Pathol 130:443-

454.

33. Trotter J, DeJong LA, Smith ME (1986): Opsonization with anti-myelin antibody increases the uptake and intracellular metabolism of myelin in inflammatory macrophages. J Neurochem. 47:779-789.

34. Smith ME, DeJong LA (1987): Antibody to myelin constituents. A possible factor in induction of cell-mediated demyelination. Neurochem Res. 12:167-172.

35. Sadler RH, Sommer M, Forno LS, Smith ME (1991): Induction of anti-myelin antibodies in EAE and their possible role in demyelination. J Neurosci Res. 30:616-624.

36. Hayes GM, Woodroofe MN, Cuzner ML (1988): Characterization of microglia isolated from adult human and rat brain. J Neuroimmunol. 19:177-189.

37. Ceuppens JL, Baroja ML, VanVaech F, Anderson CL (1988): Defect in Membrane expression of high affinity 72 KD Fc receptors on phagocytic cells in four healthy subjects. J Clin Invest 82:671-578.

38. Debets JM, Van der Linden CJ, Dieteren IE, Leuwenberg JF, Buurman WA (1988) Fc receptor cross-linking induces rapid secretion of tumor necrosis factor (cachectin) by human peripheral blood monocytes. J Immunol 141:1197-1201.

39. Tripathi AK, Taplits M, Puri J, Hoffman T (1991): Downregulation of surface FcR1 and decrease in antibody-dependent cellular cytotoxicity of cultured monocytes. Reversal by monensin or cytochalasin-D. J Immunol 146: 1309-1315.

40. Van de Winkle JGJ, Anderson CL (1991): Biology of human immunoglobulin G Fc receptors. J Leukocyte Biol 49:511-524.

41. Brown MS, Goldstein JL (1983): Lipoprotein metabolism in the macrophage: Implications for cholesterol deposition in atherosclerosis. Ann Rev Biochem 52:223-261.

42. Giulian D, Baker TJ (1986): Characterization of ameboid microglia isolated from the developing mammalian brain. J Neurosci. 6:2163-2178.

43. Norton WT (1981): Myelin in RW Albers, GJ Siegel, R Katzan, and BW Agarath (ed). Basic Neurochemistry 2nd Ed, Little Brown, Boston MA. 365-386.

44. Lee K-D, Hong K, Papahadjopoulos D (1992): Recognition of liposomes by cells: in vitro binding and endocytosis mediated by specific lipid headgroups and surface charge density. Biochim Biophys Acta 1103: 185-197.

45. Kim YS, Kim SU (1991): Oligodendroglial cell death induced by oxygen radicals and its protection by catalase. J Neurosci Res 29:100-106.

46. Chan PH, Schmidley JN, Fishman RA, Longar SM (1984): Brain injury, edema and vascular permeability changes induced by oxygen-derived free radicals. Neurology 34:315-320.

47. Debets JMH, Van de Winkle JGJ, Ceuppens JL, Dieterenm IEM, Buurman WA (1990): Cross linking of both Fc gamma RI and Fc gamma RII induces secretion of tumor necrosis factor by human monocytes, requiring high affinity Fc-Fc gamma R interactions. J Immunol 144:1304-1310.

48. Krutmann J, Kirnbauer R, Kock A, Schwarz T, Schopf E, May LT, Seehgal PB, Luger TA (1990): Cross linking Fc receptors on monocytes triggers IL-6 production. Role in anti-CD3-induced T cell proliferation activation. J Immunol. 145:1337-1342.

49. Aloisi F, Care A, Borsellino G, Gallo G, Rosa S, Bassni A, Cabibbo A, Testa U, Levi G, Peschle C. (1992): Production of hemolymphopoietic cytokines (IL-6, IL-8, colony stimulating factors) by normal human astrocytes in response to IL-1ß and tumor necrosis factor-α. J Immunol. 149:2358-2366.

50. Sebire G, Demilie E, Wallen C, Hery C, Devergne O, Delfraisoy JF, Galanaud P, Tardieu M (1993): In vitro production of IL-6, IL-1ß, and tumor necrosis factorα by human embryonic microglial and neural cells. J Immunol 150:1517-1523.

51. Hofman FM, Hanweber RG, Dinarrello CA, Mizel SB, Hinton D, Merril JE (1986):Immunoregulatory molecules and IL-2 receptors identified in Multiply Sclerosis Brain. J Immunol 136: 3239-3245.

52. Selmaj KW, Raine CS (1988): Tumor necrosis factor mediates myelin oligodendrocyte damage in vitro. Ann Neurol 23:339-346.

53. Selmaj K, Raine CS, Farooq M, Norton WT, Brosnan CF (1991): Cytokine cytotoxicity against oligodendrocytes. Apoptosis induced by lymphotoxin. J Immunol. 147:1522-1529.

54. Giulian D, Young DG, Woodward J, Brown DC, Lackman LB (1988): Interleukin-1 is an astroglial growth factor in the developing brain. J Neurosci 8: 709-714.

55. Lee SC, Liu W, Dickson DW, Brosnan CF, Berman JW (1993): Cytokine production by human fetal microglia and astrocytes. Differential induction by lipopolysaccharide and IL-1β. J Immunol 150:2659-2667.

56. Perry VH, Andersson PB, Gordon S (1993) Macrophages and inflammation in the

central nervous system. TINS 16:268-272.

Table 1. Oxidative burst by cells phagocytosing myelin

	Microglia	Astrocytes	THP-1
Cells alone	0	0	0
PMA 10 nm	97±8	4±2	80±4
PMA 100 nm	ND	41±7	ND
Myelin vesicles	7±4	1±1	22±7
with anti-MBP IgG	21±5	2±1	37±7
with human serum	69±14	2±2	35±4

Nitroblue tetrazolium reduction during the oxidative burst produces a blue precipitate within the reactive cell. Microglia, astrocytes, and THP-1 cells were incubated for 30 min at 37 degrees C with NBT and either PMA, myelin vesicles, or myelin vesicles that had been opsonized with anti-MBP mAb or heat-inactivated human serum. At the end of the assay coverslips were examined by light microscopy and results reported as the percentage of cells with reduced NBT in their cytoplasm. 100 cells per coverslip were counted. Results represent n=2 experiments of triplicated coverslips.

Table 2. Cytokine production by CNS glial cells phagocytosing myelin

	IL-1 (pg/ml)	IL-6 (pg/ml)	TNF(pg/ml)
Mixed glial cultures			
Basal	ND	33±15	8±0
Maximal	ND	719±22	213±50
Microglia			
Basal	45±11	132±92	10±6
Maximal	4656±1683	6997±2003	3813±1759
Astrocytes			
Basal	0	35±15	0
Maximal	3±2	194±46	161±60

Cytokine production induced by non-opsonized myelin uptake. The basal and maximal production of IL-1, IL-6, and TNF over a 24 hr time is reported. Data represent the mean cytokine production of triplicate wells from $4X10^4$ mixed glial cells, and $1X10^4$ enriched microglial or astrocyte cultures, ± sem. n=3 experiments.

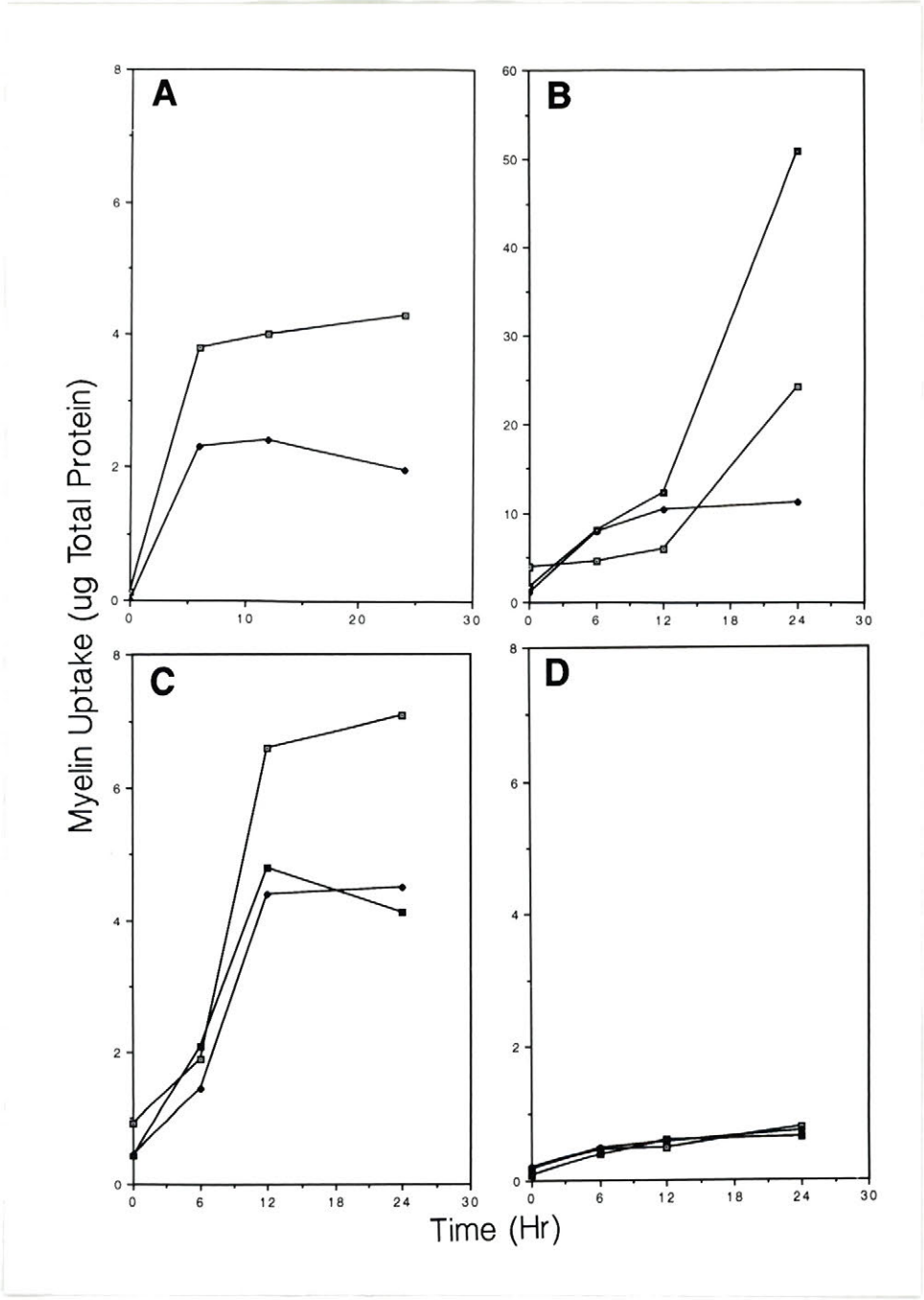

Figure 1. Time course of myelin phagocytosis by mixed adult human derived glial cells (A), human monocytic cell line, THP-1 (B), enriched adult human microglia (C) and enriched fetal human derived astrocytes (D). ^{125}I-labelled myelin vesicles were either non-treated (—●—) or opsonized with heat-inactivated human serum (—▣—) or with anti-MBP mAb (—■—) The amount of phagocytosis was determined by cell associated radioactivity after removal of myelin vesicles. CPM was converted to μg of myelin protein to account for differences in radiolabelling between experiments. Values are the mean of n=3 experiments and the SEM < 0.78 (A); 2.9 (B); 1.5 (C) and 0.2 (D).

Figure 2. Phagocytosis of FITC-labelled MBP by enriched adult human microglial cells. The internalization of MBP was determined at 37° C (A) and 4°C (B). After extensive washes fluorescein was detected at 488 nm on a Lieca Laser Tecnick Microscope. The photomicrograph demonstrates a slice through the middle of the cells indicating internal fluorescence. Phagocytosis of MBP was only detected at 37°C whereas only low background fluorescence could be detected at 4°C (Mag 400X).

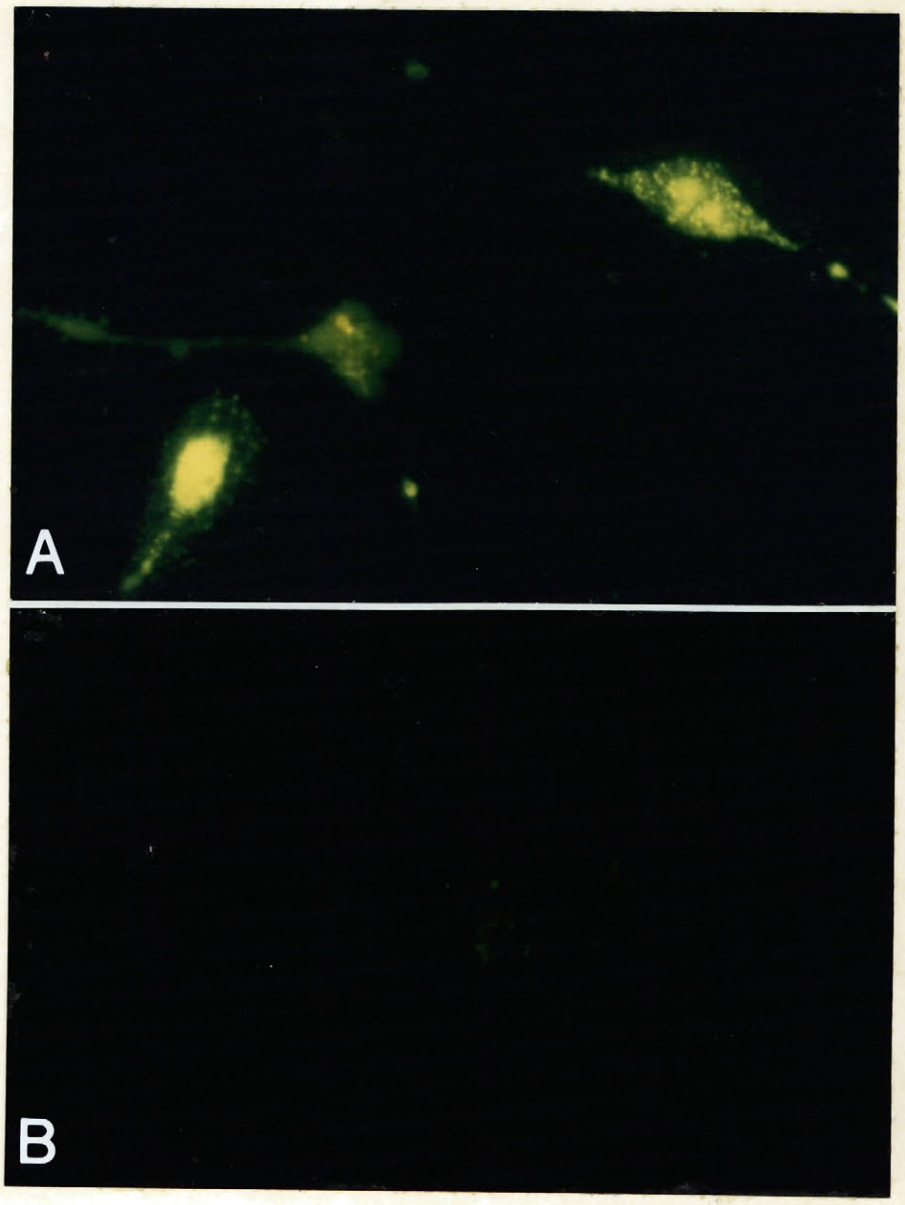
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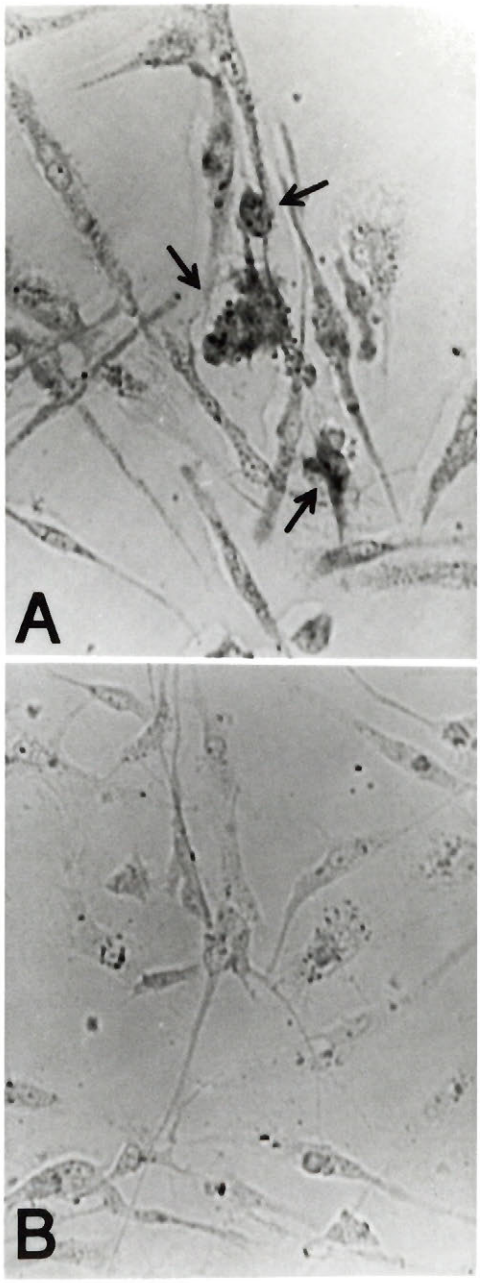
Figure 3. Oxidative burst response of microglia. Oxidative burst as determined by the reduction of NBT in a 30 min assay could be detected (arrows) in microglia incubated with myelin that had been opsonized with an anti-MBP antibody (A) but not seen in cells alone (B) (Mag 400X).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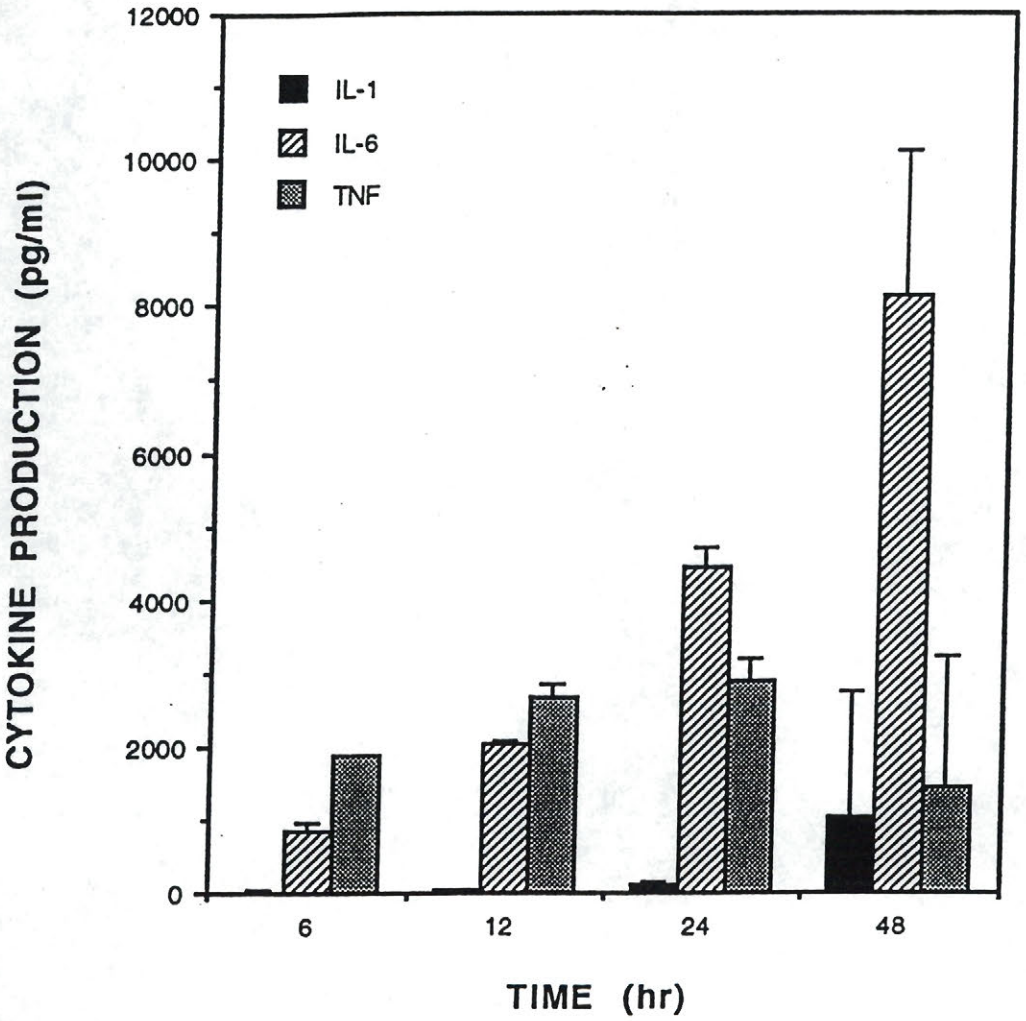

Figure 4. Microglia cytokine production as a result of myelin phagocytosis. Enriched adult human derived microglial cultures ($1X10^4$ cells per well) were incubated with myelin vesicles over a 48 hr time period. Activation of microglia was monitored by the production of IL-1 (■), IL-6 (▨), and TNF (▩) by cytokine bioassays. Data presented here are representative of N=4 experiments.

Part IV

Conclusion

Chapter 10

Conclusion and Claims to Originality

Results presented in this thesis demonstrate the ability to isolate and culture adult human derived microglia. Immunocytochemical and immunohistochemical data support the notion that microglia are most likely of bone marrow origin and are similar phenotypically to other monocyte/macrophages. Similar to resident macrophages in other tissue, the microglia studied were heterogenous both with respect to cells derived from the same tissue specimens, and with regard to variations between different donor tissues. The author acknowledges that the microglia studied in vitro are most likely activated cells, a function of both the source of the tissue and the isolation technique. Despite the inherent problem of microglial activation in vitro, within the parameters investigated, it was possible to both upregulate microglia function using IFN gamma, and to inhibit microglial functions using anti MHC class II blocking mAbs, anti-B7/BB-1 mAbs, or by blocking microglial FcRs with immune complexes. Parameters of microglia activation seen in vitro, including upregulated MHC class II and FcR expression, were also demonstrated in situ on MS brain specimens.

In addition to the ability of microglia to function as immune accessory cells to stimulate T cell activation, and to function as an immune effector cell through FcRs and IgGs, data was also presented suggesting the role that microglia might play in the elaboration of cytokines including IL-1, IL-6, and TNF, and the ability of microglia to undergo oxidative burst. Data presented in this thesis suggest that in MS microglia can contribute to ongoing pathology linking the cellular, T cell mediated arm of the immune system with the humoral, B cell arm, resulting in augmentation of CNS inflammatory events.

Claims to Originality

Chapters 2-9 contain original work, either published (Chapter 2: **J Neuropath Exp Neurol 51:**538-549, 1992, Chapter 4: **J Neurosci Res 36:**382-390, 1993; **Ann Neurol 32:**99A, 1992, Chapter 7:**J Neuropath Exp Neurol 53** (in press), Chapter 8: **J Neurol Sci** (in press), Chapter 9: **J Neurosci Res** (in press), **J Neurochem 61 (suppl):**S228A) or submitted for publication (Chapters 3,5,6).