ANTIGEN-SPECIFIC INTERACTIONS OF MACROPHAGES AND $CD4^+$T CELLS IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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ABSTRACT

Samuel J. Madison. ANTIGEN-SPECIFIC INTERACTIONS OF MACROPHAGES AND CD4+ T CELLS IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (Under the direction of Dr. Mark Mannie) Department of Molecular Biology, DEC. 1997.

Experimental autoimmune encephalomyelitis (EAE) is an animal model for the human demyelinating disease multiple sclerosis (MS). Previous experiments have indicated that macrophages participate in EAE as primary end-stage effector cells by mediating phagocytosis of CNS myelin in an antigen-specific manner. Macrophages supposedly attack the myelin sheath causing tissue damage and paralysis, but leave the rest of the axon intact. After a few days the paralysis subsides. A paradox arises here because macrophage effector action is believed to be largely nonspecific. If this were true, macrophages would not attack only the myelin sheath, but the whole axon, and there would be no recovery from paralysis. The theory tested in this thesis is that macrophages acquire T cell receptor (TCR) from T-helper cells to specifically guide macrophage-mediated phagocytic activity. Peritoneal macrophages (PEC) from Lewis rats were studied to determine whether they were recovering TCR from $CD4^+$ guinea pig myelin basic protein (GPMBP)-specific T cell lines and to address the role of nitric oxide (NO) in the acquisition process. The interaction between PEC and T cells was studied by measuring the T cell incorporation of $[^3H]$ thymidine and secretion of IL-2 with proliferative assays, and by measuring the production of NO by macrophages with the Griess reaction. Flow cytometric analysis was utilized to detect T cell and macrophage-specific markers. Myelin basic protein (MBP) presentation by high densities of PEC elicited T cell-mediated IL-2 production without accompanying proliferation, and the subsequent production of nitric oxide by PEC. The lack of T cell proliferation was attributed to cell death, which was mediated by the production of toxic levels of nitric oxide by PEC. Active sensitization of Lewis rats confirmed that NO production was highest at peak disease. The production of NO was determined to be antigen-specific. Macrophages produced high concentrations of NO only when cultured with both T cells and their specific antigen GPMBP. IL-2 was shown to inhibit this antigen-specificity. After pretreatment of macrophages with IL-2, production of NO was no longer dependent on the addition of GPMBP. Most importantly, flow cytometric analysis implied that macrophages in the presence of guinea pig myelin basic protein-specific T cells and (GPMBP) were incorporating T cell receptor. All of these data point to an effector role for macrophages in EAE, where acquisition of the antigen receptor complex of T cells engender the antigen-specific targeting of the myelin sheath.

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TABLE OF CONTENTS

LIST OF TABLES .... **vi**

LIST OF FIGURES .... **vii**

LIST OF ABBREVIATIONS .... **ix**

1 INTRODUCTION 1

1.1 Overview of MS and EAE 1

1.2 A Role for Nitric Oxide in the Etiology of EAE 2

1.3 Peripheral Mechanisms of Tolerance Induction of T cells 7

1.4 Interaction of T cells and Macrophages 9

2 MATERIALS AND METHODS **12**

2.1 Animals 12

2.2 Reagents 12

2.3 Derivation of T cell Lines 13

2.4 T cell Activation 13

2.5 Preparation of PEC 14

2.6 Preparation of SPL 14

2.7 Measurement of IL-2 Production and Proliferation 14

2.8 Measurement of Nitric Oxide Production 15

2.9 SNP Assay 16

2.10 Adoptive Transfer 16

2.11 Biotinylation 16

2.12 Preparation of *C. Parvum* 17

2.13 Sub-diploid PI Method 17

2.14 Analysis of T cells (3 Color Flow) 18

2.15 Analysis of PEC 18

3 RESULTS **20**

3.1 Analysis of the Interaction Between Antigen Presenting Cells and T cells Reveals a Variation in T cell Response 20

3.2 Flow Cytometric Analysis Demonstrates Cell Death and Subsequent Disappearance of T cells 23

3.3 Nitric Oxide is the Mediator of Cell Death 28

3.4 The Production of Nitric Oxide by PEC is Antigen-Specific 35

3.5 IL-2 Affects Inducibility and Antigen-Specificity of NO production by Macrophages 41

3.6 The Incorporation of TCR by PEC 41

3.7 A Possible Function of the Incorporation of TCR by PEC 52

3.8 NO Production in EAE 53

3.9 Adoptive Transfer Studies Suggests a More “Active” Role For PEC in EAE 53

4 DISCUSSION ………………………………………………………. **61**

4.1 SPL and PEC React Differently in the Presence of T-Helper Cells 61

4.2 T Cells Are Undergoing Cell Death Mediated By PEC ………….. 61

4.3 Nitric Oxide is Mediator of T Cell Death ………………………… 62

4.4 Antigen Specificity of NO-Mediated T Cell Death ………………. 64

4.5 The Acquisition of T Cell Receptor by Macrophages ……………. 65

4.6 A Function for the Binding of TCR ………………………………. 67

5 REFERENCES ……………………………………………………... **69**

LIST OF TABLES

1. Antigen-Specificity of NO Production In Splenocytes 38

2. Nitric Oxide Has Marginal Effects on the Incorporation Of TCR by PEC 51

3. Comparative APC Activity of IrrSPL and IrrPEC For Adoptive Transfer of EAE 57

LIST OF FIGURES

1. Reaction Catalyzed By Nitric Oxide Synthase 4

2. Proliferation vs. IL-2 Production in T Cells Cultured With PEC and SPL 22

3. Evidence of T Cell Death By the Subdiploid PI Method ... 25

4. Downregulation of T cell Markers 27

5. NMA Augments Proliferation of T Cells in a Dose-dependent Fashion 30

6. Aminoguanidine Augments Proliferation of T Cells and Inhibits NO Production By PEC 32

7. NMA But Not NAC Inhibits Death of T Cells 34

8. The Production of NO By SNP Inhibits Proliferation of T Cells 37

9. Antigen-Specific Production of NO by PEC 40

10. IL-2 Pre-treatment of Macrophages Inhibits Antigen-Specific NO Production 43

11. IL-2 Pre-treatment of Macrophages Elevates Baseline NO Production But Inhibits Antigen-Induced NO Production... 45

12. Initial evidence of Incorporation of T Cell Receptor by Macrophages 48

13. Further Evidence of Incorporation of TCR by Macrophages 50

14. Irradiated T Cells Can Reactivate Resting PEC as Evidenced By NO Production 55

15. TCR-Positive Macrophages Acquire Antigen-Specificity 57

16. Nitric Oxide Production is Optimum at Peak Disease 60

LIST OF ABBREVIATIONS

APC	Antigen Presenting Cell
CFA	Complete Freund's Adjuvant
CM	Conditioned Medium
CNS	Central Nervous System
cRPMI	Complete RPMI
CTL	Cytotoxic T Lymphocyte
EAE	Experimental Autoimmune Encephalomyelitis
GPMBP	Guinea Pig Myelin Basic Protein
HBSS	Hank's Balanced Salt Solution
IFN	Interferon
IL	Interleukin
irr	Irradiated
LPS	Lipopolysaccharide
MBP	Myelin Basic Protein
M-CSF	Monocyte Colony Stimulating Factor
MHC	Major Histocompatability Complex
MS	Multiple Sclerosis
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NRS	Normal Rat Serum

OD	Optical Density
PEC	Peritoneal Exudate Cell(s)
PI	Propodium Iodide
SNP	Sodium Nitroprusside
SPL	Splenocyte(s)
TCR, Tcr	T cell Receptor
Th	T Helper Cell
TNF	Tumor Necrosis Factor

Chapter 1

INTRODUCTION

1.1 Overview of MS and EAE

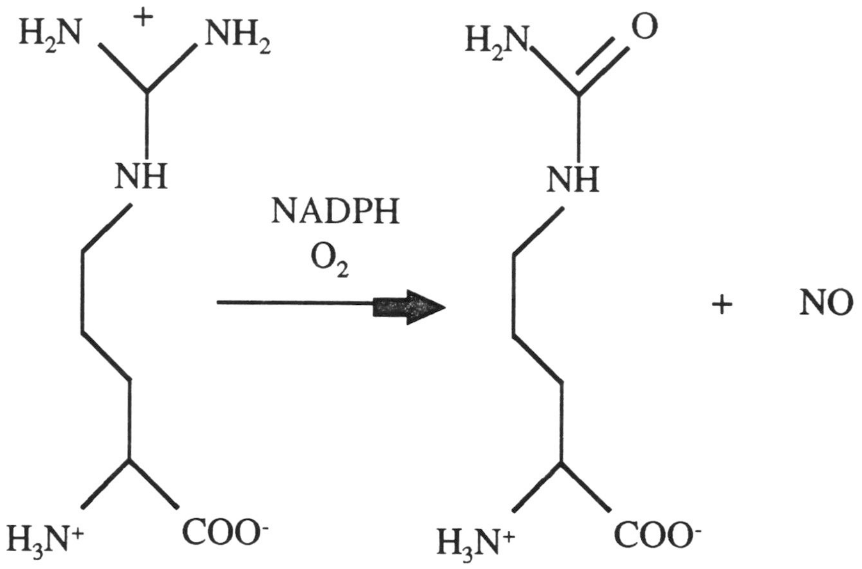
Multiple sclerosis (MS) is a relapsing or progressive inflammatory demyelinating disease of the central nervous system (CNS) in which the myelin sheath and the myelin-producing cells, oligodendrocytes, are destroyed. Myelin is composed of lipids and protein, forming a cover around axons to increase the conduction of nerve impulses. Although the etiology of MS is still unclear, pathological studies have indicated that cells infiltrating the white matter of the brain are mostly composed of activated T cells and macrophages which produce proinflammatory cytokines such as TNF-alpha and IFN-gamma (Immura et al., 1993). In particular, T cell autoreactivity to myelin basic protein (MBP), a major protein constituent of CNS myelin, figures prominently in the pathology of the disease (Sinha et al., 1990).

Experimental Autoimmune Encephalomyelitis (EAE), an animal model of MS, is a cell-mediated autoimmune response directed against autologous CNS myelin. The CNS is continuously surveyed by the immune system. $CD4^+$ T cells which are activated in the circulation can pass through the blood-brain barrier irrespective of their specific target antigens. Most of these T cells will not find their specific antigen and will migrate from the CNS parenchyma. However when T cells that are directed against an autoantigen of the CNS (MBP), confront their antigen on APCs in the perivascular space, they will be further activated leading to a cascade of secondary events, such as upregulation of the production of cytokines and adhesion molecules, and further induction of the expression of MHC class II antigens on local APCs. These events facilitate the entrance of a secondary wave of leukocytes into the CNS leading to disease and local tissue damage. Sensitization of Lewis rats with GPMBP-specific $CD4^+$ T cells cultured with GPMBP and APCs mediate the transfer of EAE.

1.2 A role for nitric oxide in the etiology of EAE

Peritoneal exudate cells (PEC) harvested from Lewis rats are comprised of 30-50% T cells and roughly 30% macrophages. High densities of macrophages are known to produce large quantities of nitric oxide in the presence of GPMBP and $CD4^+$ T cells. Nitric oxide (NO) is a potent effector molecule which is involved in a number of physiological activities, ranging from the regulation of vascular tone and neurotransmission to the killing of microbes and tumor cells (Kitajima et al., 1994). Nitric oxide is synthesized by the enzyme nitric oxide synthase (NOS), which catalyzes the oxidation of L-arginine to NO and citrulline (Fig. 1). Three isoforms of NOS enzymes have been characterized, two of which are constitutively expressed and a third which is inducible (Albina et al., 1993). The constituitive NOS enzymes (cNOS), neuronal NOS (NOS I) and endothelial NOS (NOS III), are calcium calmodulin dependent and produce small amounts of NO. The inducible NOS (iNOS, NOS II), originally described in macrophages, is expressed by cells following transcriptional induction, thus the transcriptional regulation of iNOS controls the production of NO (Kitajima et al., 1994). Type II NOS firmly binds calmodulin at resting Ca^{2+} concentrations, so its activity is calcium independent (DeVera et al., 1996). Nitric oxide is formed at much higher rates by iNOS than by cNOS. Inducible NOS, which is typically not present in unstimulated cells, has been proposed to have a major influence

Figure 1. Reaction catalyzed by nitric oxide synthase. Nitric oxide is synthesized by the enzyme NOS from an L-arginine substrate to citrulline.


H2N
+
NH2
NH
H3N+
COO-
NADPH
O2
H2N
O
NH
H3N+
COO-
+
NO

L-arginine citrulline nitric oxide

in the inflammatory process, and expression is regulated by inflammatory cytokines including IL-1, TNF-alpha, IFN-gamma, and the endotoxin lipopolysaccharide (LPS) (Kitajima et al., 1994). Nitric oxide produced by iNOS promotes the activation of guanylyl cyclase, inhibits enzymes (aconitase, ribonucleotide reductase, ADP-ribosylation of proteins), metabolic pathways (mitochondrial respiration, DNA synthesis), and through these actions, participates in a vast array of processes, including host defense, autoimmunity, and rejection of engrafted tissues (Kroemer and Martinez, 1995).

Nitric oxide in excess amounts is cytotoxic (Cross et al., 1996, Hooper et al., 1997, Ruuls et al., 1996). The mechanisms by which NO mediates toxicity are not fully understood, but among its cytotoxic actions are inhibition of several enzymes of the electron transport chain and the citric acid cycle by its reaction with the iron-sulfur centers of proteins (Hewett et al., 1996). Nitric oxide can produce toxicity through its interaction with the free radical, superoxide, by forming the highly toxic substance, peroxynitrite anion (Zhao et al., 1996), which kills cells, inhibits mitochondrial respiration, and leads to lipid peroxidation (Cross et al., 1996, Hooper et al., 1997, Ruuls et al., 1996). Nitric oxide activates cyclooxygenase-2, an inducible enzyme responsible for production of prostaglandin E_2, which contributes to EAE lesion development (Cross, et al., 1996).

Nitric oxide has been shown to be toxic to cultured oligodendrocytes, the cells that produce CNS myelin (Merrill, 1993). Evidence suggests a role for NO in the upregulation of TNF-alpha, an inflammatory cytokine with myelinotoxic properties implicated in the pathogenesis of EAE (Cross et al., 1996). Nitric oxide can also upregulate MHC class II expression in antigen-specific $CD4^+$ T cell recognition, and enhance chemotaxis, supporting a role for NO in recruitment of additional cells to sites of inflammation (Cross et al., 1996). Macrophages constitute a large proportion of the inflammatory cells that infiltrate the CNS of animals with EAE. Macrophages can contribute to the regulation of the immune reaction through the production of inflammatory mediators such as NO. Several papers have backed this idea. Macrophages isolated from the CNS of Lewis rats with active EAE have been shown to produce elevated levels of NO (Ruuls et al., 1996). Increased mRNA encoding iNOS was detected in EAE-affected CNS tissue using reverse transcriptase polymerase chain reaction assays (rtPCR). Spinal cord mRNA expression was at maximal levels during early acute EAE when inflammation was at its highest (Cross et al., 1996). Okuda and co-workers, using an immunohistochemical examination of mouse spinal cord with an iNOS-specific antibody showed the frequency of iNOS-positive cells to be highest at peak disease. These iNOS-positive cells were composed of infiltrating macrophages that were mostly located in the necrotic area (Okuda et al., 1995). Thus direct and indirect evidence argue that NO in the CNS is likely to be pathogenic and pro-inflammatory.

Yet many other studies have demonstrated an immune-suppressive role for NO in EAE (Pender et al., 1991, Ruuls et al., 1996, Tabi et al., 1995), where autoreactive cells are killed by NO, which aids in clearing of the disease. The basis of this idea is that autoreactive T cells, oligodendrocytes, and myelin are being destroyed in the CNS during EAE. At the same time, infiltrating macrophages are producing high concentrations of NO which has been determined to be highly toxic to the cells and tissue in question. It would be reasonable to concur that NO would be the culprit in this case. The system, however, is much more complex than previously thought. And even if NO is the cause of cell death and inflammation in EAE, there is still much skepticism surrounding the role that NO plays in Multiple Sclerosis.

1.3 Peripheral mechanisms of tolerance induction of T cells

Physical elimination of self-reactive T cells during their maturation in the thymus is the main mechanism for establishment of self-tolerance (Arnold et al., 1993). The majority of these cells are eliminated by clonal deletion, but it is now apparent that many also escape intrathymic deletion and emerge into the periphery. These potentially dangerous cells must subsequently be suppressed or eliminated by the less well characterized process of peripheral T cell tolerance (Munn et al., 1996). It has been shown clearly that distinct events can take place in the periphery leading to nonresponsiveness, including ignorance of self antigen, anergy, and perhaps deletion.

The term ignorance describes circumstances in which a cell with some potential antiself-reactivity fails to react to the self-antigen in question. It is inevitable that some interactions between lymphocyte receptors and antigen will fall just short of the threshold for activation or tolerance induction. However, if there is an increase in antigen concentration or a rise in the number of receptors per cell, a lymphocyte that might previously have ignored the self antigen might now respond to it. Furthermore, some self-antigens may be sequestered inside cells or behind anatomical barriers which would make them incapable of interacting with lymphocyte receptors. If cell or tissue damage occurred, interaction would now become possible (Nossal, 1994).

In the case of anergy, mature lymphocytes present in the peripheral lymphoid organs become functionally inactivated, possibly by the interaction with antigen in the absence of co-stimulatory signals necessary for generation of an immune response (Kuby, 1994). Phenotypic appearance, and a resistance to reactivation in vitro characterize T cell anergy. Recently, evidence has emerged suggesting a third tolerogenic pathway involving the antigen-specific deletion of peripheral T cells. The research presents an *in vitro* model of that very pathway, where autoreactive T cells are specifically deleted by macrophages through the induction of NO mediated apoptosis.

Macrophages have been shown to induce the selective, antigen-specific deletion of T cells (Munn et al., 1996). The mechanism responsible for the lack of proliferation is either anergy, apoptosis, or necrosis of the cells. Anergy, as I have previously stated, is a state of inactivation marked by the inability of T cells to proliferate in response to an antigen-MHC complex. Cell death (irreversible loss of vital cellular structure and function) is a fundamental phenomenon of biological organisms. Cell death occurs as a physiologic process during organogenesis in embryos, and in cell turnover in adults as both a normal physiologic event, and a pathologic process in response to various injuries. Two distinct mechanisms for cell death have been identified, namely, necrosis and apoptosis (Albina et al., 1993, Buja et al., 1993).

A major difference between apoptosis and necrosis is that apoptosis is programmed cell death. Necrosis is not deliberately programmed; it follows injury in which the cell is mechanically disrupted, or in which homeostasis is compromised, for example, by toxins, low pH, or anoxia (Cohen, 1993). The earliest changes visible in cells undergoing necrosis are in the mitochondria, which swell and accumulate calcium phosphate crystal deposits in their lumens. At this point, the mitochondria can no longer make sufficient ATP to control ion pumps; water enters down an osmotic gradient, and the cell bursts causing disruption of the mitochondrial and plasma membranes (Cohen, 1993, Mitrovic et al., 1995). This liberation of cytoplasmic organelles into the surrounding tissue results in random DNA cleavage, an immune response, and local inflammation (Bauer et al., 1995).

Apoptosis is considered to be the major process responsible for cell death in both embryonic tissue remodeling and adult cell turnover and differentiation. Apoptosis is characterized by nuclear and cytoplasmic condensation of single parenchymal cells (shrinkage), loss of nuclear membrane, and fragmentation of DNA into oligonucleotides 180 to 200 bp in length (Albina et al., 1993, Buja et al., 1993, Mitrovic et al., 1995). The usual fate of an apoptotic cell is to be phagocytosed by the nearest cell capable, allowing tidy disposal before the release of proinflammatory degradation products (Bauer et al., 1995, Cohen, 1993).

1.4 Interaction of T cells and Macrophages

T cells and macrophages interact in a number of physiological processes and are primary in both humoral and cell-mediated responses . Recognition by T cells is carried out by a heteromeric T cell receptor that is generated in a manner similar to immunoglobulins. During T-helper cell maturation, progenitor T cells migrate from the bone marrow to the thymus where random rearrangement of germ line DNA gene segments takes place, producing a diverse system of antigen receptors (Kuby, 1994). Each T cell clone expresses uniquely rearranged TCR gene elements and expresses a homogeneous clonotypic population of TCR to confer unique antigen specificity. Each T cell is able to recognize specific antigen on the surface of macrophages, which leads to T cell activation. In the first phase of this process, antigen presentation, antigen is engulfed by macrophages, processed, and expressed on their surface of the macrophage in association with MHC class II. T cells bearing specific antigen receptors for this antigen can then bind to the antigen/MHC complex, leading to activation, proliferation and the clonal expansion of T-helper cells specific for the same antigen. These activated T cells can, in turn, activate macrophages and by unknown mechanisms, guide macrophage effector activity. So T cell activation is dependent upon macrophage antigen presenting activity and macrophage effector activity relies on signals from T cells.

Macrophages and T cells are both thought to play a role in EAE. Experimental autoimmune encephalomyelitis can be passively induced in Lewis rats by the transfer of activated MBP-specific T cells. The autoimmune attack, characterized by a progressive paralysis from distal tail to hind legs, is followed by spontaneous recovery. It has been proposed that the down-regulation of EAE is due to the deletion of autoreactive T cells by macrophages (Tabi et al., 1995). Infiltrating macrophages are thought to allow T cell activation and expression of T cell effector function as expressed by CD3 down-modulation, IL-2 and IL-2 receptor up regulation but inability to respond to IL-2, while selectively inhibiting T cell proliferation (Uphan et al., 1984). But macrophages are also suspected of carrying out the effector role in EAE by actively destroying the axonal extensions of the myelin sheath. Yet nonspecific macrophages would not destroy only the myelin sheath, but the whole axon, and there would not be clinical recovery.

Many studies have demonstrated that cells of the monocyte-macrophage lineage function in primary demyelination and tissue damage through the activation of these inflammatory cells (Brosnan et al., 1981). Both resident and infiltrating blood-born macrophages are present in EAE lesions of the CNS, but the resident macrophages, due to their strategic position, appear to be more effective in removal of myelin debris (Rinner et al., 1995). Recent studies have suggested that macrophages are activated in patients with MS and that they may contribute to the demyelinating process through their production of cytokines such as TNF-alpha, IL-1, and IL-6 (Imamura et al., 1993). This targeted activity by macrophages is hard to explain on the basis of known functions of effector T cells. Epstein and associates concluded that ligand-mediated phagocytosis could explain the targeted activity of macrophages in EAE (Epstein et al., 1983), where the destruction of intact myelin sheaths by macrophages involves the attachment of myelin lamella to coated pits on the macrophage surface prior to endocytosis. But a central paradox arises here. The idea that non-specific macrophages can specifically target and destroy the myelin sheath, causing paralysis, yet not destroy the whole axon, allowing recovery from clinical signs of EAE is difficult to explain. This paradox is the basis of the following research where the incorporation of T cell surface proteins by macrophages may lead to the antigen-specific targeting of the myelin sheath.

Chapter 2

MATERIALS AND METHODS

2.1 Animals

Lewis rats (Harlan Sprague-Dawley, Indianapolis, IN) were bred in our animal facility and were 6 to 18 weeks of age when used in experiments. Rats received a standard rat chow and water *ad libitum.*

2.2 Reagents

MBP was purified from guinea pig spinal cords (Pel-Freez, Rogers, AR) as described previously (Swanborg, 1988). Sodium Nitroprusside, N^G-nitro-L-arginine, N^G- monomethyl-L- arginine, Ribonuclease A, LPS, CFA, Con A, cycloheximide, and propidium iodide were obtained from Sigma Chemical Co. (St. Louis, MO). R73 (IgG1 anti-Tcr), ED2 (IgG2 anti-macrophage), and PE-conjugated (goat anti-mouse IgG1) mAbs were obtained from Pharmingen (San Diego, CA). OX19 (IgG1 anti-CD5) and W3/25 (IgG1 anti-CD4) mAbs were obtained from Sera-Lab. FITC (goat anti-mouse IgG2a) mAb came from Southern Biotechnology (Birmingham, AL). ED1 (IgG1 anti-macrophage) mAb was purchased from Serotec. FITC-conjugated $F(ab')_2$ rat anti-mouse mAb came from Accurate Chemical (Westbury, NY). FAV IV and B36 mAbs were gifts from Dr. A. Mason Smith. Aminoguanidine hydrochloride was obtained from Tocris Cookson (St. Louis, MO). Sulfo-NHS-LC-Biotin was ordered from Pierce (Rockford, IL). Streptavidin Red-670 came from Life Technologies (Grand Island, NY). *C. parvum* was a gift from Dr. Robert S. Fulghum. MTS came from Promega (Madison WI).

2.3 Derivation of T cell lines

The polyclonal R2 T cell line and the R2.2F4 T cell clone (Vß4$^+$) were isolated from rats sensitized with 10 nmoles of R72-86 (^{72}PQKSQ-RTQDENPV^{86}) (Mannie, et al., 1996) in CFA. R2.2F4 T cells were isolated by limiting dilution cloning of R2 T cells at 0.5 cells/well. Cell culture was performed in complete RPMI 1640 medium (cRPMI; Life Technologies, Grand Island, NY), 10% heat-inactivated fetal bovine serum (Sigma Chemical Co., St. Louis, MO), 2 mM glutamine, 100 µg/ml streptomycin 100 U/ml penicillin (Whittaker Bioproducts, Walkersville, MD), and 50 µM 2-ME (Sigma Chemical Co.). Conditioned medium (CM) was cRPMI supplemented with a source of recombinant IL-2.

2.4 T cell activation

T cells were activated for use in proliferative assays, IL-2 production assays and flow cytometric analysis. T cells were washed in HBSS, and activated with irradiated splenocytes (irrSPL), and 2 µM GPMBP in cRPMI. Cultures were maintained in 37°C incubators at 5% CO_2. After specified times, cells were harvested and used in assays.

2.5 Preparation of Peritoneal Exudate Cells (PEC)

Peritoneal exudate cells were activated by 48 hour sensitization of Lewis rats with 5 ml of 40 µg/ml heat killed *Corynebacterium parvum.* PEC were harvested by rinsing the peritoneum with 50 ml of Hank's balanced salt solution (HBSS) (GIBCO BRL, Gaithersburg, MD). These PEC were washed three times in HBSS and irradiated with 3000 rads of gamma-irradiation from a Gammator-50 source of ^{137}Cs. Following irradiation, PEC were harvested by centrifugation, washed once again in HBSS, and resuspended in cRPMI.

2.6 Preparation of SPL

Spleens from untreated Lewis rats were aseptically dissected and pressed through stainless steel wire mesh screens to obtain a suspension of cells. These SPL were washed three times in HBSS and irradiated to 3000 rads of gamma-irradiation from a Gammator-50 source of ^{137}Cs. Following irradiation, splenocytes were harvested by centrifugation, washed once again in HBSS, and resuspended in cRPMI.

2.7 Measurement of IL-2 production and proliferation

T cells (2.5 x 10^4/well) were cultured in the presence or absence of irradiated SPL or PEC, with or without 2 µM GPMBP and nitric oxide inhibitors (AG, NMA or NNA) for 48 hours. Cells were pulsed with 0.5 µCi [^{3}H]thymidine during the last 24 hours of culture. After culture, the supernatants (100 µl/well) were transferred into replicate plates, and the T cells were then harvested on fiberglass filters using a Mach III M cell harvester (Tomtec, Orange, CT), and counted on a 1450 Mircobeta Plus liquid scintillation counter (Wallac, Gaithersburg, MD) to measure [^{3}H]thymidine incorporation. To determine IL-2 activity of culture supernatants, IL-2-dependent CTLL cells (5 x 10^4/50 µl/well) were cultured with 100 µl of T cell supernatants for 24 hours. During the last 4 hours of culture, 20 µl (10 mg/ml) of MTS (Sigma) (Mannie et al., 1996) were added to each well to measure viability and growth of the CTLL cells. The plates were then read at 570 nm on an Anthos ELISA reader (model 2001;ACCSales, Chapel Hill, NC). By this procedure, IL-2 production and proliferation were measured in the same single well cultures. MBP-stimulated IL-2 production was calculated as the mean OD values from MBP-stimulated cultures minus mean OD values from control, unstimulated cultures.

2.8 Measurement of nitric oxide production

The production of nitric oxide (NO), as measured by formation of the stable decomposition product nitrite, was determined in cell-free supernatant using a microplate assay described by Ding and co-workers (Ding et al., 1988). PEC or SPL (1 x 10^6/well) were plated in the presence or absence of 2 µM GPMBP and various NO inhibitors for 48 hours. Supernatant (50 µl) was mixed with 50 µl Griess reagent (1% sulfanilamide plus 0.1% N-[1]-naphtylethylenediamine in 2.5% H_3PO_4). After 10 min incubation at room temperature, the optical density at 540 nm was measured in a microplate reader (model 2001;ACCSales, Chapel Hill, NC).

2.9 Sodium Nitroprusside (SNP) assay

T cells (2.5 x10^4/well) were incubated in the presence of 0.4% IL-2 and the nitric oxide donor SNP (10 mM-78 μM) in a 96 well microtiter plate for 48 hours. Proliferation and nitric oxide production were assayed (Kong, et al., 1994).

2.10 Adoptive transfer

PEC were elicited by i.p. injection of 200 μg *C. parvum* in 5 ml Hank's buffered saline solution and were recovered after 2 days from Lewis rats. MBP-specific T cells (2.5 x 10^6/ petri dish) were cultured with 25 x 10^6 irrSPL or irrPEC in the presence or absence of 2 μM GPMBP. After 2 days of culture, cells were extensively washed and injected i.v. into Lewis rat recipients. The following scale was used to assign intensity of EAE; paralysis in the distal tail, 0.25; limp tail, 0.5; ataxia, 1.0; hindleg paresis, 2.0; full hindleg paralysis, 3.0. The mean maximal intensity was the average maximum score among afflicted rats within a group.

2.11 Biotinylation

Cells were first washed three times in HBSS. Pellet (10 x 10^6 cells/ml) was incubated in freshly prepared 1 mM Sulfo-NHS-LC biotin (Pierce) with shaking 30 min at room temperature (2 hours at 4°C),then washed three more times in HBSS. Strept Red-670 was added as a secondary stain at .5 μl /sample (see flow protocol).

2.12 Preparation of *C. Parvum* (*Propionibacterium Acnes*)

The bacterial sample was reconstituted in PYG (Pepsin Yeast Extract and Glucose + Tween 80), added to chopped meat medium and incubated for 48 hours at 37° C. Boiling distilled water was then added to 1 L and the sample was gassed with CO_2 while cooling. Cystine was then added to the starter culture, which was capped and autoclaved. The bacterial sample was added to the starter culture and incubated for 48hours. The growth medium was capped, autoclaved and allowed to cool. The starter culture was then added and incubated for 48 hours. After incubation, the culture was heat-killed at 56°C for 30 minutes, centrifuged at 5000 rpm for 10 minutes, washed two times in PBS and once in distilled water. The sample was brought up in the respective amount of distilled water, shell-freezed, lyophilized, weighed and stored at -80°C. The bacterial sample can be reconstituted in PBS with 1:1000 Thimerosal, at 7 mg/ml.

2.13 Sub-diploid Propidium Iodide (PI) method

Irradiated adherent SPL and PEC (20 x 10^6/dish) were incubated with T cells (10 x 10^6/dish), 2 μM GPMBP with or without 316 μM NMA in tissue culture dishes for 48 hours. Cells were washed twice in HBSS supplemented with 5% calf serum and 0.02% sodium azide (HSA) and incubated for 20 minutes in 20 μl normal rat serum (NRS). The samples were then stained with 100 μl of primary antibody (R73 TCR-specific antibody or ED1 macrophage-specific antibody) for 45 min. Cells were washed twice in HSA, incubated again in NRS, and then with 100 μl of secondary antibody (FITC-conjugated $F(ab')_2$ rat ant-mouse) for 45 min. Washed cells were slowly vortexed while adding 1 ml of ice cold 70% ETOH and were incubated over night. Ethanol was poured off and 100 µl of RNase A (1 mg/ml) was added while vortexing. Samples were then incubated with 100 µl of Propidium Iodide (PI) for 30 min. Samples were analyzed by the production of histograms on a Becton Dickinson 440 flow cytometer (BD, Mountain View, CA). Percent apoptotic cells are measured as sub-G1 cell population present.

2.14 Analysis of T cells (3 color flow)

Irradiated PEC or SPL (20 x 10^6/dish) were incubated with and without T cells, 2 µM GPMBP and 316 µM NMA (NO inhibitor) in tissue culture dishes at 37° C for 48 hours. Dishes were scraped, and cells were resuspended in HBSS supplemented with 5% calf serum and 0.02% sodium azide. The cells were incubated with 10% normal rat serum (HSA) for 20 min at 4° C followed by the addition of one of the following mAb: R73 (anti-TCR), OX19 (anti-CD5), ED2 (macrophage-specific mAb), OX19 and ED2, and R73 and ED2 for 45 min. Cells were washed, incubated again in NRS followed by addition of FITC-conjugated (goat anti-mouse IgG2a) and/or PE-conjugated (goat anti-mouse IgG1) for 45 min. and were analyzed on a Becton Dickinson 440 flow cytometer (Mountain View, CA).

2.15 Analysis of PEC

PEC were rested for 48 hours in cRPMI and 20% monocyte-colony stimulating factor (M-CSF). (Hoedemakers et al., 1988). M-CSF was obtained by culturing L929 cells in cRPMI which were allowed to grow to comfluence. The cells were separated from the supernatant by centrifugation and supernatants were then filtered and used as a source of M-CSF. Adherent cells were irradiated, biotinylated and cultured with and without T cells, AG and GPMBP for 48 hours. Cells were resuspended and incubated with NRS followed by addition of one of the following mAb: LRTC1 (anti-LFA-1), FAV IV and B36 (irrelevant, isotype-matched controls), R73 (anti-TCR) ED1 (macrophage-specific mAb), and W3/25 (anti-CD4). Cells were washed, incubated again in NRS followed by addition of both FITC-conjugated $F(ab')_2$ rat ant-mouse and streptavidin Red-670 and were analyzed by flow cytometer.

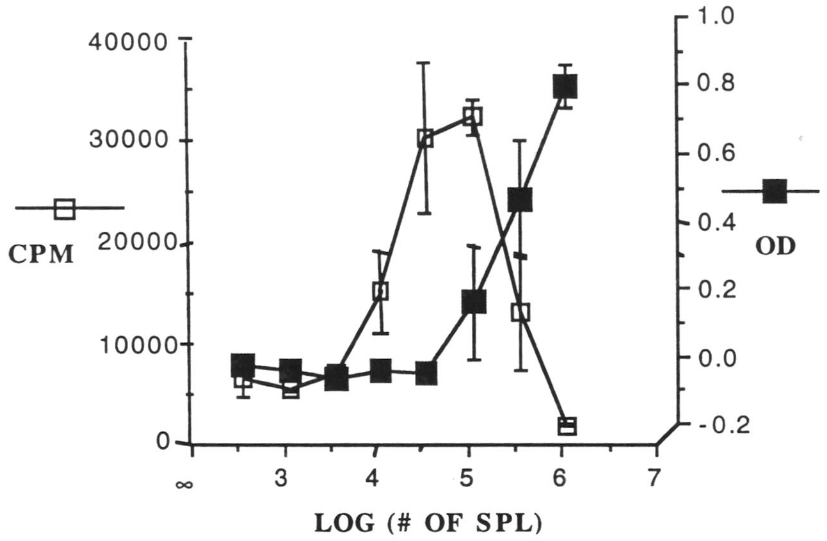
Chapter 3

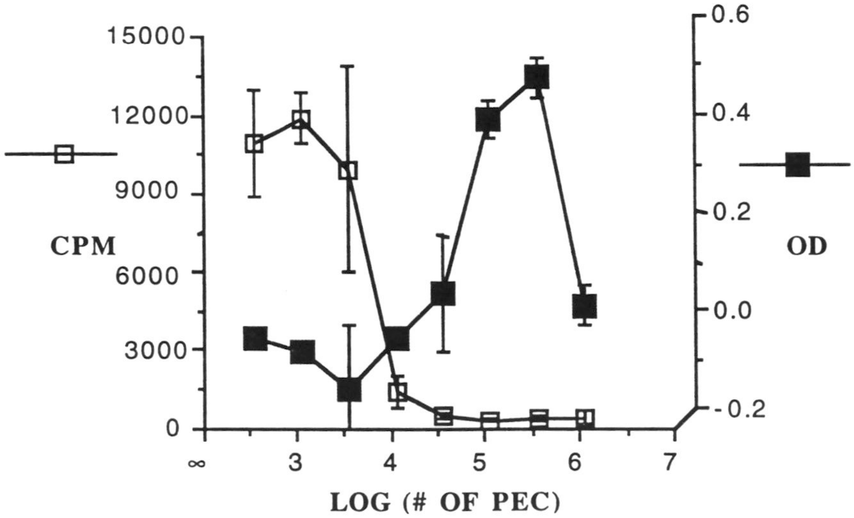
RESULTS

3.1 Analysis of the interaction between antigen presenting cells and T cells reveals a variation in T cell response.

The purpose of this study was to assess the interaction between T cells and two different types of APC. Splenocytes are known antigen presenting cells. When GPMBP-specific T cell lines (2.5 x 10^4/well) are incubated with irr SPL (1 x 10^6/well) in the presence of antigen (2 μM GPMBP), they proliferate and produce high levels of IL-2. Proliferation and IL-2 production follow a bell-shaped curve as the concentration of SPL increases. Antigen presentation by macrophages induces the activation of the T cell, blastogenesis and the production of various cytokines. One would think substituting PEC, a source of professional APC, for SPL would elicit similar results. But low concentrations of peritoneal macrophages stimulate antigen-dependent T cell growth. As the density of PEC increases, proliferation decreases drastically although IL-2 production is spared except at high densities of PEC where the response drops dramatically (Fig. 2). What may occur is that the peritoneal macrophages present antigen to the T cells which in turn produce IL-2, but the macrophages then kill the T cells by unknown mechanisms.

Figure 2. Proliferation vs. IL-2 production in T cells cultured with PEC and SPL. T cells (2.5 x 10^4/ well) were cultured with irr PEC or irr SPL (1 x 10^6/ well) with and without 2 μM GPMBP for 48 hours. **Proliferation (CPM).** Supernatants were transferred to replicate plates, and T cells were harvested and counted to measure [^{3}H]thymidine incorporation. **IL-2 production (OD).** To determine IL-2 activity, CTLLs (5 x 10^4/ 50 μl/ well) were cultured with supernatants for 24 hours. During the last 4 hours of culture, 20 μl of MTT were added to each well to measure viability and growth. The left vertical axis represents proliferation and the right vertical axis represents IL-2 production. These data are representative of at least two experiments.


40000
30000
20000
10000
0
CPM
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
OD
∞
3
4
5
6
7
LOG (# OF SPL)


15000
12000
9000
6000
3000
0
CPM
0.6
0.4
0.2
0.0
-0.2
OD
∞
3
4
5
6
7
LOG (# OF PEC)

3.2 Flow cytometric analysis demonstrates cell death and subsequent disappearance of T cells

With the observation that T cell proliferation was inhibited in the presence of macrophages, the next step was to determine the mechanism causing the inhibition of proliferation. Apoptosis, or programmed cell death, is characterized by a pronounced decrease in cell volume, membrane blebbing, condensation of chromatin, and degradation of DNA into oligonucleosomal fragments which are cells with less than the diploid amount of DNA (Pender et al., 1991). The subdiploid PI flow cytometric method was used to confirm cell death. Using this technique, dead cells were measured as sub-G1 phase or cells with less than the diploid amount of DNA. Results show a significant increase in subdiploid cells in the absence of NMA, a nitric oxide inhibitor (Fig. 3). Further experiments with flow cytometric analysis compared cultures of resting T cells to T cells in the presence of irr PEC and irr SPL. Cells exhibiting the scatter properties of T cells were isolated and assayed for the presence of various cell surface markers. Resting T cells and T cells cultured with irr SPL expressed high fluorescence for the monoclonal antibodies OX19 (anti-CD5) and R73 (anti-TCR), where T cells cultured with irr PEC had very low readings for both markers (Fig. 4). All of this taken together would suggest that T cells in the presence of PEC are being killed and then engulfed by the macrophages.

Figure 3. Evidence of T cell death by the subdiploid PI method. Irradiated adherent PEC (20 x 10^6/dish) were incubated with T cells (10 x 10^6/dish), 2 μM GPMBP, with and without 316 μM NMA in tissue culture dishes for 48 hours. Cells were fixed in 70% ETOH. RNase A (1 mg/ml) was then added, followed by staining with 400 μg/ml propidium iodide and analysis on flow cytometer. Percent dead cells were measured as sub-G1 cell population present. These data are representative of two experiments.

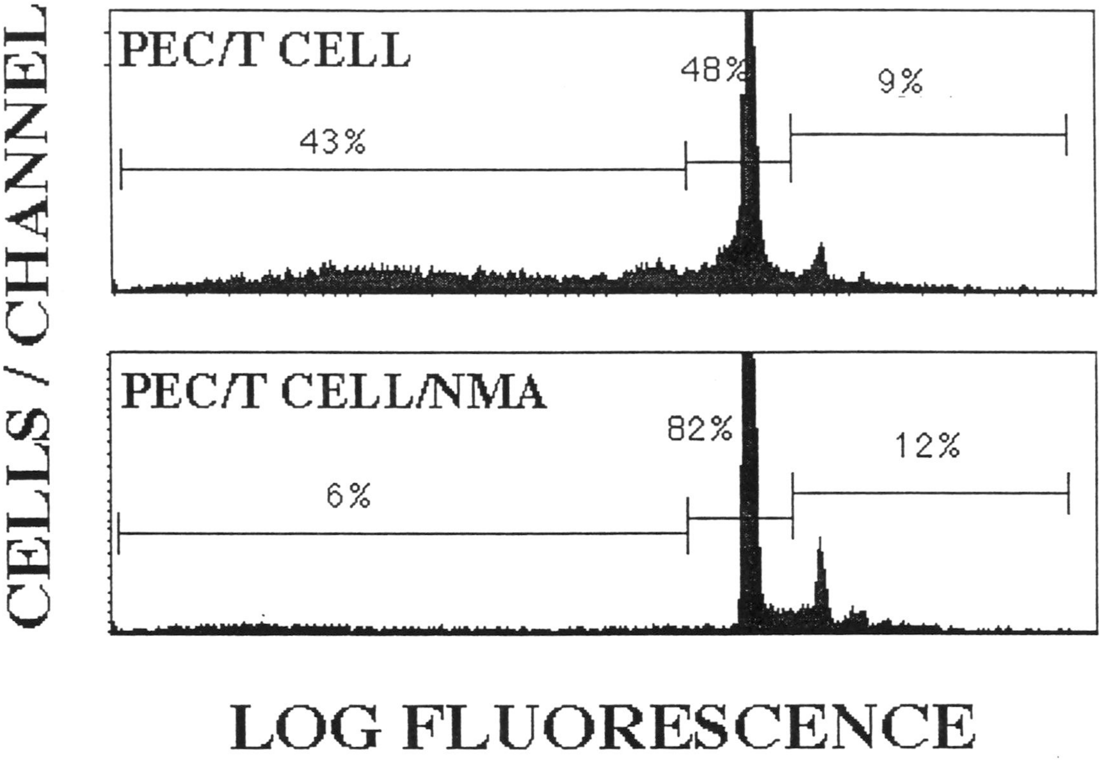
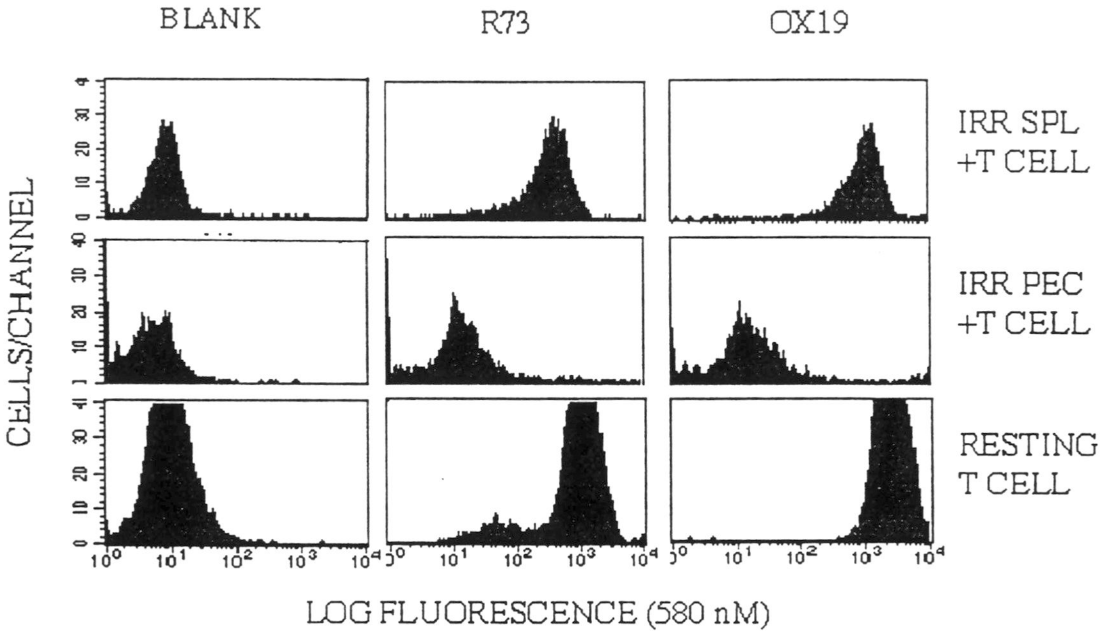

PEC/T CELL
48%
9%
43%
PEC/T CELL/NMA
82%
12%
6%
CELLS / CHANNEL
LOG FLUORESCENCE

Figure 4. Downregulation of T cell markers. T cells (2 x 10^6/dish) were incubated with irradiated adherent SPL or PEC (20 x 10^6/dish), 2 μM GPMBP with and without 316 μM NMA in tissue culture dishes for 48 hours. Cells were stained with R73, OX19, and ED2 plus PE goat anti-mouse or FITC goat anti-mouse and analyzed on a flow cytometer. These data are representative of two experiments.


BLANK
R73
OX19
IRR SPL +T CELL
IRR PEC +T CELL
RESTING T CELL
CELLS/CHANNEL
0
10
20
30
40
10^0
10^1
10^2
10^3
10^4
LOG FLUORESCENCE (580 nM)

3.3 Nitric oxide is the mediator of cell death

Nitric oxide (NO) is a potent effector molecule which is involved in a number of physiological activities, ranging from the regulation of vascular tone and neurotransmission, to the killing of microbes and tumor cells (Kitajima et al., 1994). Nitric oxide production by activated macrophages is a common phenomenon proposed to have a major influence on the inflammatory process. To test the possibility that nitric oxide was the agent responsible for the death of T cells, the effect of several nitric oxide inhibitors on the system was measured. N^G-momomethyl-L-arginine (NMA) is a competitive irreversible inhibitor of iNOS which is the enzyme that catalyzes the production of NO in macrophages, and a reversible inhibitor of cNOS (Hewett et al., 1996, Orucevic and Lala, 1996, Zhao et al., 1996). Aminoguanidine (AG) is a selective inhibitor of iNOS. N^G-nitro-L-arginine (NNA) is an inhibitor of cNOS. The results, using proliferative assays and measured spectrophotometrically as nitrite formed during the exposure using the Griess reaction showed that NMA and AG abrogated NO production in a dose-dependent fashion, allowing the T cells to proliferate (Fig. 5 and 6). NNA had very little effect on this system compared to the other inhibitors (Data not shown). Experiments comparing NMA with N-acetylcysteine (NAC), a thiol antioxidant which inhibits activation-induced T cell death mediated by oxidative stress (Sandstrom et al., 1994), showed that NAC had little effect on the inhibition of T cell proliferation compared to NMA (Fig. 7). Tests were also performed using the NO

Figure 5. NMA augments proliferation of T cells in a dose-dependent fashion. PEC were plated with T cells (2.5 x 10^4/well), 2 μM GPMBP, and various concentrations of NMA for 48 hours. **Measurement of proliferation.** Supernatants were transferred to replicate plates, and T cells were harvested and counted to measure [^{3}H]thymidine incorporation as described in Materials and methods. These data are representative of two experiments.

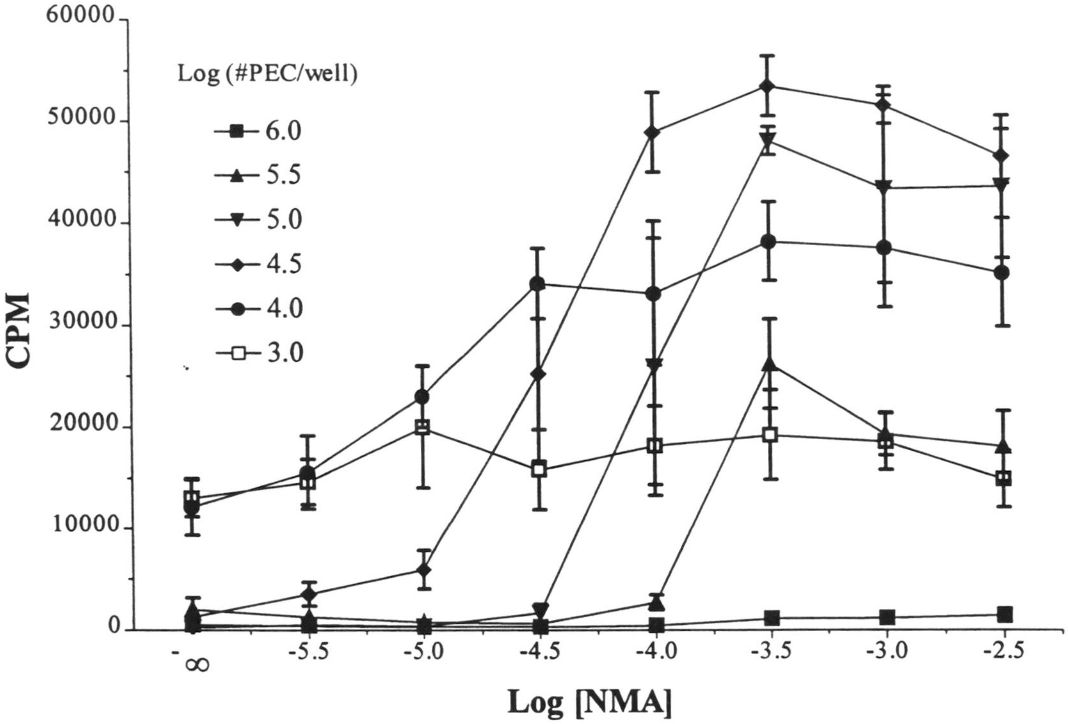
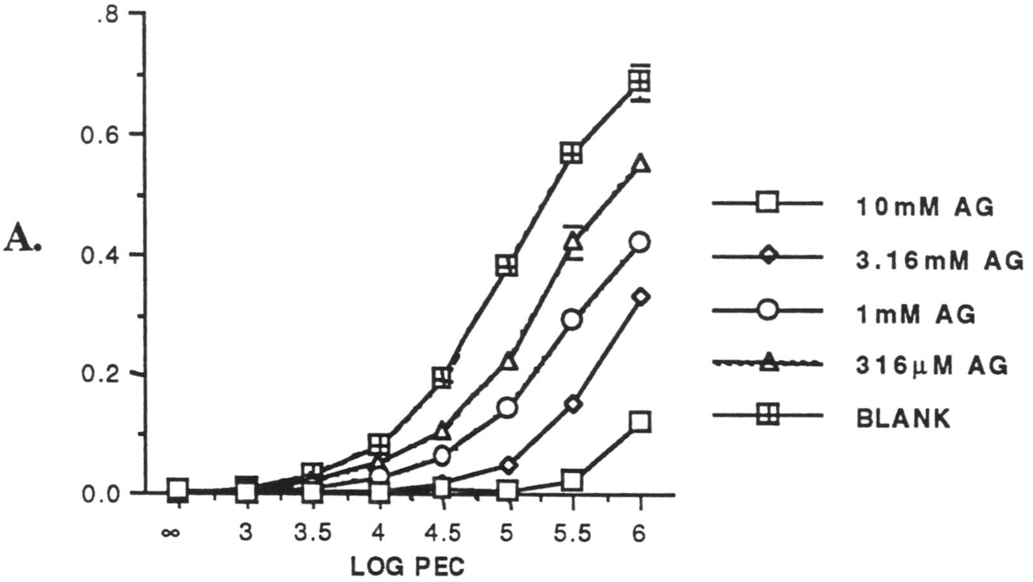

CPM
60000
50000
40000
30000
20000
10000
0
Log (#PEC/well)
6.0
5.5
5.0
4.5
4.0
3.0
- ∞
-5.5
-5.0
-4.5
-4.0
-3.5
-3.0
-2.5
Log [NMA]

Figure 6. Aminoguanidine augments proliferation of T cells and inhibits NO production by PEC. PEC were plated with T cells (2.5 x 10^4/well), 2 μM GPMBP, and various concentrations of AG for 48 hours. **B. Measurement of proliferation.** Supernatants were transferred to replicate plates, and T cells were harvested and counted to measure [^{3}H]thymidine incorporation as described in Materials and methods. **A. Nitric oxide production.** The production of nitric oxide, as measured by formation of nitrite, was determined using a microplate assay described in Materials and Methods. Supernatant (50 μl) was mixed with equal amounts of Griess reagent and read on an ELISA plate reader. These data are representative of two experiments.


A.
.8
0.6
0.4
0.2
0.0
∞
3
3.5
4
4.5
5
5.5
6
LOG PEC
10mM AG
3.16mM AG
1mM AG
316µM AG
BLANK

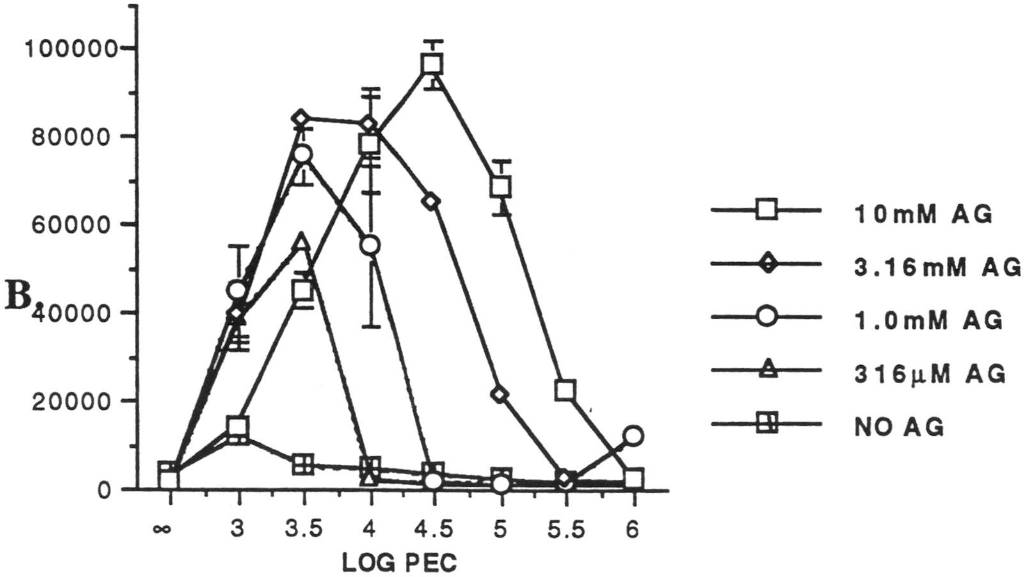
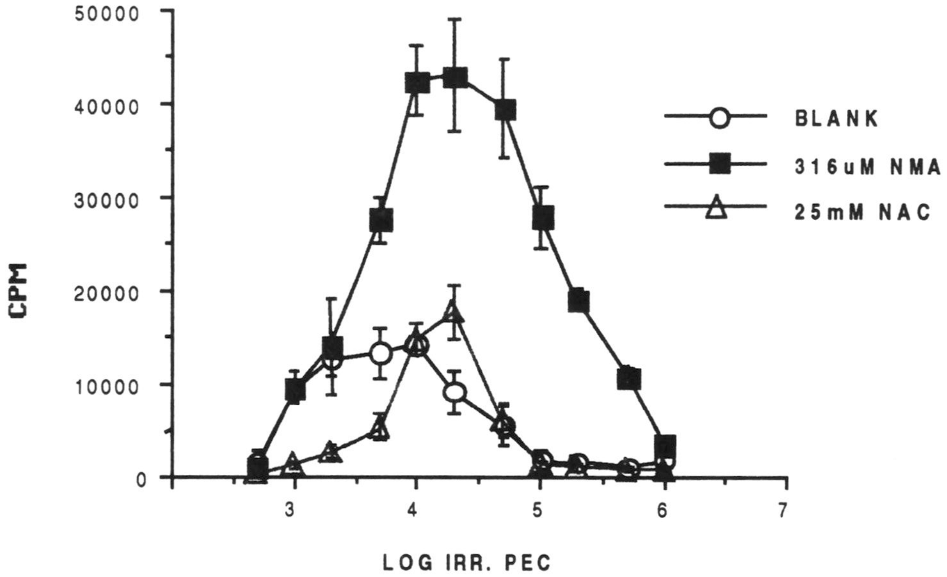

B.
100000
80000
60000
40000
20000
0
∞
3
3.5
4
4.5
5
5.5
6
LOG PEC
10mM AG
3.16mM AG
1.0mM AG
316µM AG
NO AG

Figure 7. NMA but not NAC inhibits death of T cells. Irradiated PEC were plated with T cells (2.5 x 10^4/well), with and without 316 μM NMA and 25 mM NAC, and 2 μM GPMBP for 48 hours (Sandstrom et al., 1994). **Measurement of proliferation (CPM).** Supernatants were transferred to replicate plates, and T cells were harvested and counted to measure [^{3}H]thymidine incorporation. These data are representative of two experiments.


50000
40000
30000
20000
10000
0
CPM
3
4
5
6
7
LOG IRR. PEC
BLANK
316uM NMA
25mM NAC

donor, sodium nitroprusside (SNP), on T cells in the presence of IL-2. The results verified that NO can inhibit the proliferation of T cells (Fig. 8). These data imply that the production of toxic levels of nitric oxide by T cells is the mediator of T cell death and that iNOS is the enzyme responsible for its production.

3.4 The production of nitric oxide by PEC is antigen-specific

Macrophages are blood monocytes that have differentiated and migrated to a specific tissue (Crawford, 1984). Functions of macrophages include phagocytosis, killing of microorganisms, antibody-dependent cellular cytotoxicity, the processing and presentation of antigen to lymphocytes, and the secretion of substances necessary for the initiation and regulation of the immune response (Lewis and McGee, 1992). By definition, macrophages are nonspecific cells. Their phagocytic and cytotoxic mechanisms act on what ever cell or tissue in their proximity (Marcinkiewicz et al., 1996). But in EAE, the specific destruction of the myelin sheath by macrophages must be antigen-specific. Early experiments with SPL reflected the antigen-specific production of NO. Though NO levels were too low to inhibit proliferation, it was only produced in the presence of T cells and GPMBP (Table 1). Several experiments using PEC assayed with LPS, T cells, or T cells plus GPMBP confirmed this theory by showing that peritoneal macrophages would produce high levels of NO when stimulated by LPS, or in the presence of T cells plus GPMBP (FIG. 9). T cells and GPMBP were completely synergistic (Data not shown). This data support the idea that the production of NO by macrophages from PEC and SPL is indeed antigen specific, with the antigen being myelin basic protein.

Figure 8. The production of nitric oxide by SNP inhibits the proliferation of T cells. T cells (2.5 x 10^4/ well) were incubated in the presence of 0.4% IL-2 and the designated concentrations of the NO donor SNP for 48 hours. Supernatants were transferred to replicate plates, and T cells were harvested and counted to measure [^{3}H]thymidine incorporation. The production of nitric oxide, as measured by formation of nitrite, was determined using a microplate assay described in Materials and Methods. Supernatant (50 µl) was mixed with equal amounts of Griess reagent and read on an ELISA plate reader. These data are representative of two experiments.

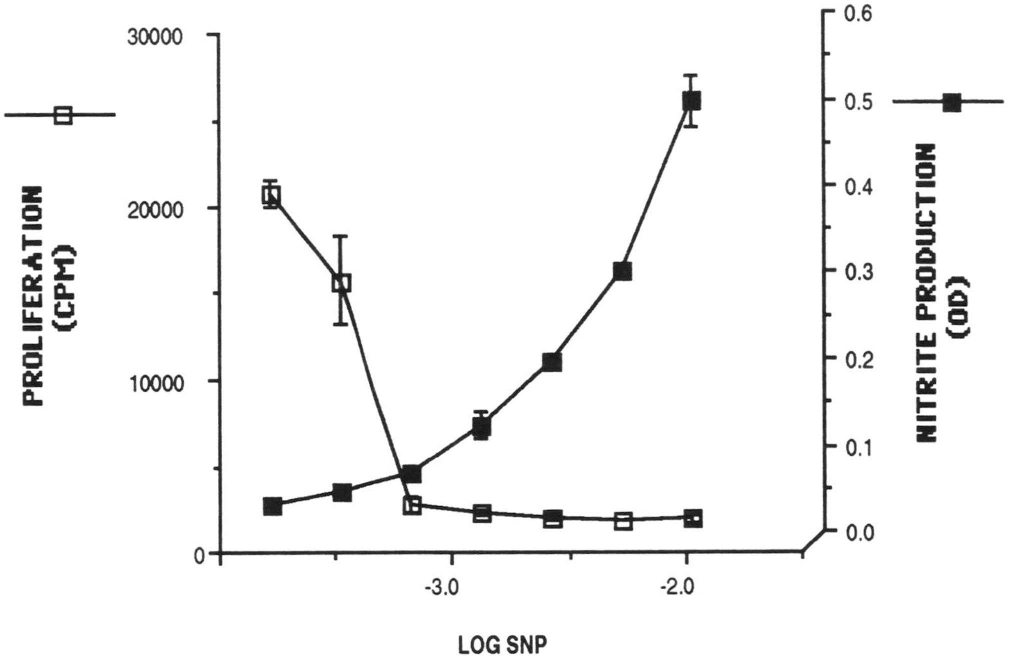

PROLIFERATION
(CPM)
30000
20000
10000
0
NITRITE PRODUCTION
(OD)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-3.0
-2.0
LOG SNP

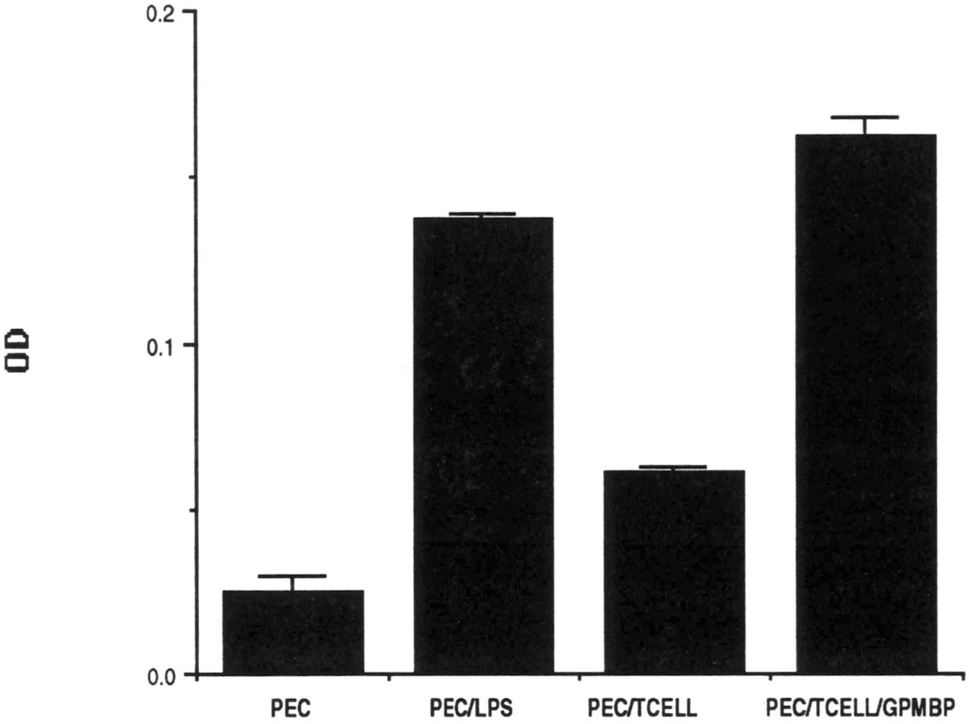
Table 1

Antigen-specificity of NO production in splenocytes.

Variables	Nitrite Production Mean	Nitrite Production SD	Proliferation Mean	Proliferation SD
SPL+GPMBP+TCELL	0.037	0.002	60122	6501
SPL+GPMBP	0.002	0.001	649	103
SPL+TCELL	0.001	0.001	896	183
SPL ONLY	0.009	0.001	953	176
TCELL ONLY	0.006	0.001	915	150

Irradiated SPL (1 x 10^6/well) were plated with and without T cell (2.5 x 10^4/well) and 2μM GPMBP for 48 hours. **Measurement of proliferation.** Supernatants were transferred to replicate plates and T cells were harvested and counted to measure [^{3}H]thymidine incorporation as described in Materials and Methods. **Nitric oxide production.** The production of NO as measured by formation of nitrite, was determined using a microplate assay described in Materials and Methods. Supernatants (50 μl/well) were mixed with equal amounts of Griess reagent and read on an ELISA plate reader.

Figure 9. The antigen-specific production of NO by PEC. PEC (2.5 x 10^5/ml) were rested in cRPMI for 48 hours. Cells were irradiated and plated with and without T cells (2.5 x 10^4/well) and 2 μM GPMBP for 48 hours. **Nitric oxide production.** The production of nitric oxide, as measured by formation of nitrite, was determined using a microplate assay described in Materials and Methods. Supernatant (50 μl) was mixed with equal amounts of Griess reagent and read on an ELISA plate reader, represented on the Y axis as optical density (OD). These data are representative of at least two experiments.


OD
0.2
0.1
0.0
PEC
PEC/LPS
PEC/TCELL
PEC/TCELL/GPMBP

3.5 IL-2 affects inducibility and antigen-specificity of NO production by Macrophages

Interleukin 2 (IL-2) is a cytokine secreted by T_H1 type $CD4^+$ T cells (Marcinkiewicz and Chain, 1993). Some of its major biological functions include inducing proliferation of antigen-primed T_H and T_C cells, supporting long-term growth of antigen-specific T cell clones, and enhancing the activity of macrophages and T_C cells (Upham et al., 1984). IL-2 is known to induce the synthesis of pro-inflammatory cytokines such as IFN-gamma, TNF, and IL-1 by lymphokine-activated killer cells (LAK) which induce NOS expression in macrophages (Yim et al., 1995). To test this on the present system, macrophages were cultured with and without IL-2 for 48 hours and assayed with either LPS (an endotoxin known to stimulate NO production in macrophages), T cells, or T cells and GPMBP. The IL-2 treated cells could not be induced to produce NO with LPS or T cells and GPMBP, where the untreated cells could (Fig. 10). Similar results were seen when IL-2 was added directly to the assay, or activated T cells, which secrete their own IL-2, were used (Fig. 11). Together these results demonstrated that IL-2 is inhibiting the antigen-specific production of NO, perhaps by the blocking of the iNOS enzyme, leaving only the constitutive amount of NO production.

3.6 The incorporation of TCR by macrophages

Previous studies have demonstrated that T cells down regulate TCR and other cell-surface proteins upon activation by APCs (Dustin et al., 1996, Rabinowitz et al.,

Figure 10. IL-2 pretreatment of macrophages inhibits antigen-specific nitric oxide production. PEC (2.5 x 10^5/ml) were rested in cRPMI with and without 0.4% IL-2 for 48 hours. Cells were irradiated and plated with and without T cells (2.5 x 10^4/well), 2 µM GPMBP, LPS (10 µg/ ml) and 3.2 mM AG for 48 hours. **Nitric oxide production.** The production of nitric oxide, as measured by formation of nitrite, was determined using a microplate assay described in Materials and Methods. Supernatant (50 µl) was mixed with equal amounts of Griess reagent and read on an ELISA plate reader, represented on the Y axis as optical density (OD). These data are representative of two experiments.

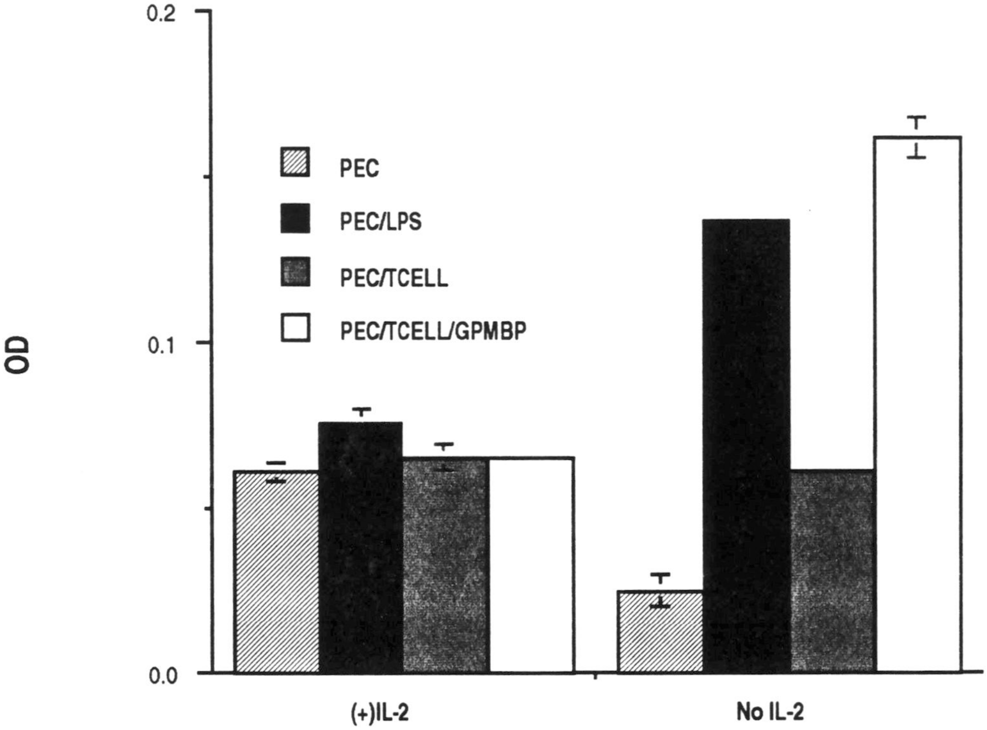
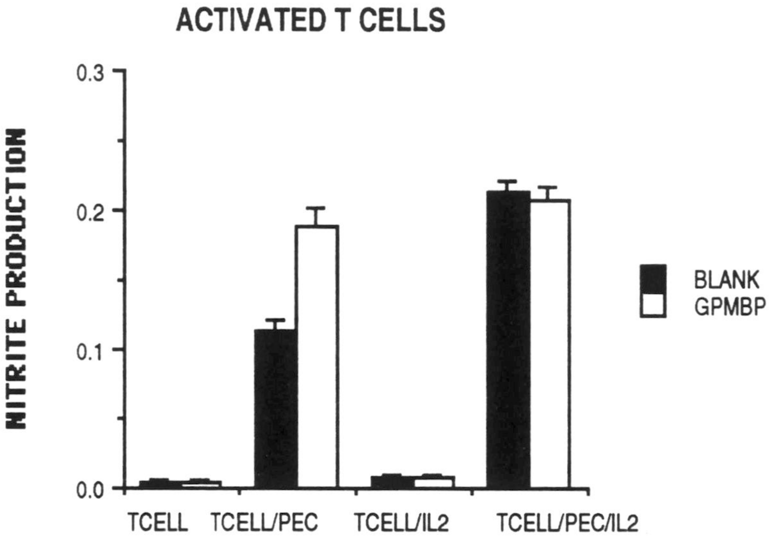
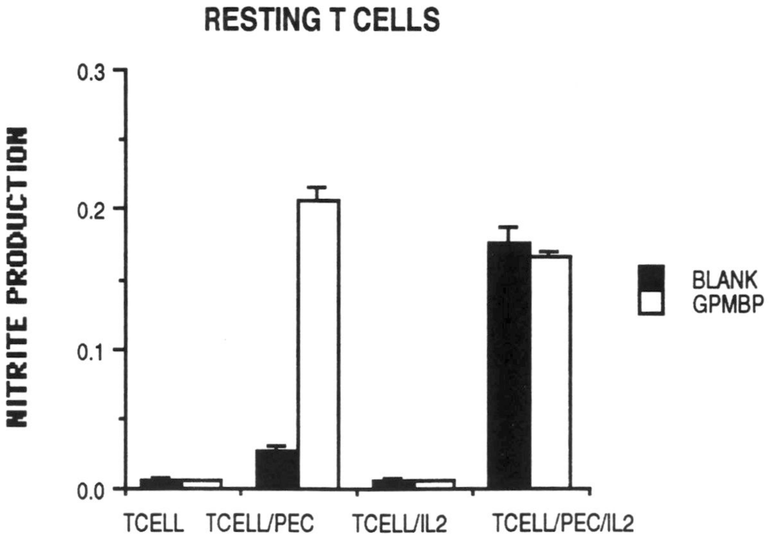

OD
0.2
0.1
0.0
PEC
PEC/LPS
PEC/TCELL
PEC/TCELL/GPMBP
(+)IL-2
No IL-2

Figure 11. IL-2 pretreatment of macrophages elevates baseline NO production but inhibits antigen-induced NO production. PEC (2.5 x 10^5/ml) were cultured in cRPMI with and without 1% IL-2 for 24 hours. Cells were irradiated and plated with activated or resting T cells (2.5 x 10^4/well), with and without 1% IL2, 2 μM GPMBP and 1 mM AG for 48 hours. **Nitric oxide production.** The production of nitric oxide, as measured by formation of nitrite, was determined using a microplate assay described in Materials and Methods. Supernatant (50 μl) was mixed with equal amounts of Griess reagent and read on an ELISA plate reader, represented on the Y axis as optical density (OD). These data are representative of two experiments.

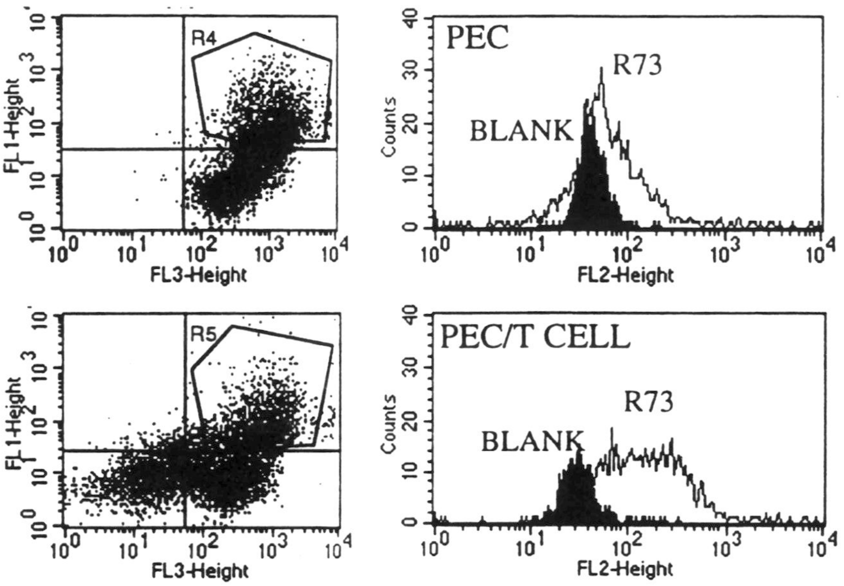

ACTIVATED T CELLS


RESTING T CELLS

1995). If T cells were losing their TCR, then the next task was to determine if these cell surface proteins were somehow being transferred to the PEC. This was accomplished by flow cytometric analysis. Early experiments using 2 color flow hinted at the existence of cells staining positive for both ED2 (macrophage-specific antibody) and R73 (data not shown). In an early 3 color flow experiment, biotinylated PEC were cultured with and without T cells and GPMBP. The PEC/T cell culture revealed a significantly larger population of biotinylated ED-2-positive cells that also exhibited high fluorescence for R73, compared to PEC alone (Fig. 12). In a confirmational experiment, resting PEC were biotinylated and cultured with and without T cells. Biotinylation of PEC allowed specific identification of macrophages during flow analysis. Exclusion of doublets (gating out cells bound to each other) ensured that a macrophage bound to a T cell would not be mistaken for a macrophage with a TCR. Visual counts were taken of each culture to verify low doublet percentages. Macrophages cultured alone showed fluorescence for R73 and the blank to be the same. Macrophages cultured with T cells exhibited a much higher fluorescence for R73 compared to the blank (Fig. 13).

Additional flow analyses were performed to determine if the presence of nitric oxide or GPMBP affected TCR incorporation (Table 2). The use of resting macrophages compared to activated macrophages did not effect the incorporation of TCR (Exp. 1 vs. Exp. 2). The addition of GPMBP or AG neither inhibited nor augmented TCR incorporation, though the addition of GPMBP did augment ED1 staining and inhibit LRTC1 staining (Exp. 3). This result was unexpected because TCR incorporation by macrophages is most likely an antigen-specific action.

Figure 12. Initial evidence of incorporation of T cell receptor by macrophages. Biotinylated PEC (20 x 10^6/dish) were cultured with and without T cells (10 x 10^6/dish), 3.2 mM AG and 2 μM GPMBP for 24 hours. Cells were then stained with ED2, R73, RED-670 streptavidin, PE goat anti-mouse and FITC goat anti-mouse as described in Materials and Methods and analyzed on flow cytometer. FL1= ED2 macrophage antibody. FL2= R73 TCR antibody. FL3= biotin/streptavidin complex (all macrophages). Macrophages staining double positive for both ED1 and biotin/streptavidin were gated on and analyzed for presence of TCR. These data are representative of two experiments.



BLANK=43
R73=94

BLANK=32
R73=206

Figure 13. Further evidence of incorporation of TCR by macrophages. PEC (20 x10^6/dish) were cultured for 48 hours in 20% M-CSF and cRPMI. Adherent cells were irradiated, biotinylated and cultured with and without T cells (10 x 10^6/dish), 3.2 mM AG and 2 μM GPMBP for 48 hours. Cells were resuspended and stained with either no antibody or one of the following mAb: LRTC1 (anti-LFA-1), R73 (anti-V-Beta), and ED1 (macrophage-specific mAb), followed by addition of both FITC-conjugated $F(ab')_2$ rat ant-mouse and streptavidin Red-670 and were analyzed by flow cytometer. Macrophage/T cell doublets, which would appear as a TCR-positive macrophage, were excluded by gating and visual counting of cultures. These data are representative of at least two experiments.

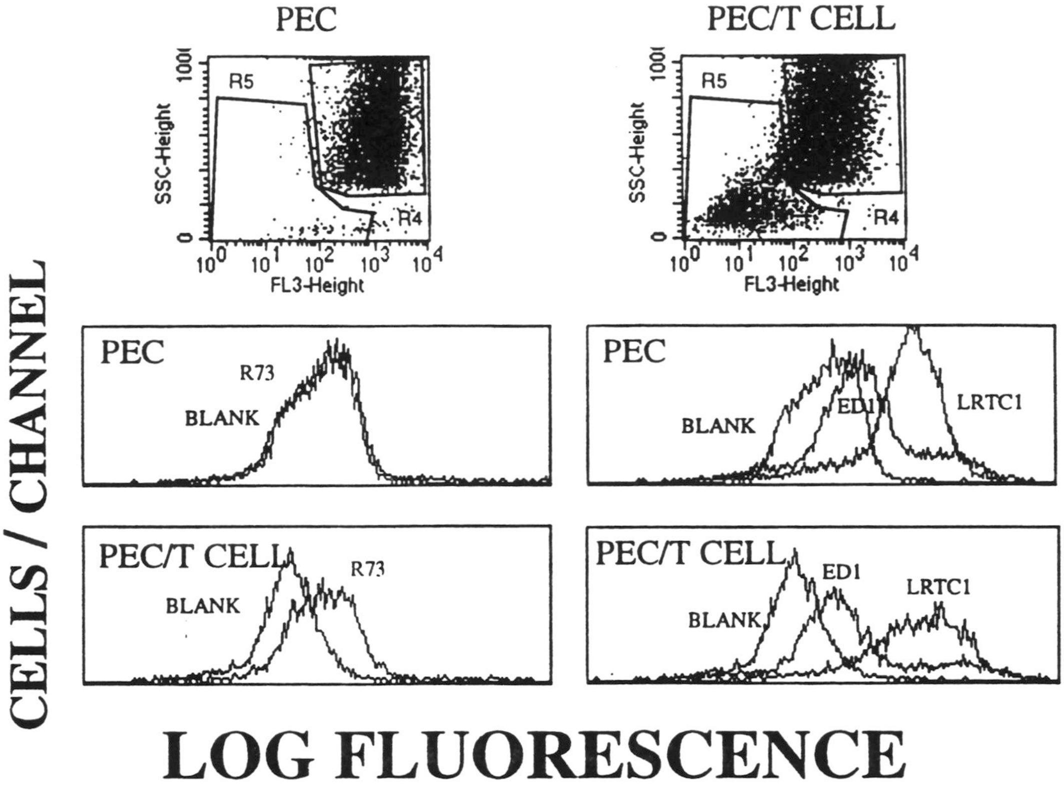

PEC
PEC/T CELL
1000
0
SSC-Height
R5
R4
10^0
10^1
10^2
10^3
10^4
FL3-Height
PEC
R73
BLANK
PEC
BLANK
ED1
LRTC1
PEC/T CELL
BLANK
R73
PEC/T CELL
ED1
LRTC1
BLANK
CELLS / CHANNEL
LOG FLUORESCENCE

Table 2
Mean Fluorescence

Condition	Blank	B36	R73	LRTC1	ED1
[a]Exp. 1					
T cell	3.4	4.0	150	248	3.5
rPEC	112	130	133	605	362
rPEC/T cell	60	67	134	752	273
[b]Exp. 2					
T cell	2.8	3.0	87	157	3.3
PEC	42	47	88	649	126
PEC/T cell	26	27	77	345	174
rPEC	94	96	115	517	176
rPEC/T cell	32	33	60	296	79
[c]Exp. 3					
T cell	2.3	2.4	69	78	2.4
PEC/T cell	13	15	35	327	16
PEC/T cell/GPMBP	13	17	41	166	83
PEC/T cell/AG	18	19	46	349	38
PEC/T cell/AG/GPMBP	10	11	48	198	27

---

[a] PEC (20 x10^6/dish) were rested for 48 hours in 20% M-CSF and cRPMI.

[b] Adherent cells and activated PEC fresh from rats were irradiated, biotinylated and cultured with and without T cells (10 x 10^6/dish), 3.2 mM AG and 2 μM GPMBP.

[c] PEC were cultured in the presence or absence of NO using NO inhibitor AG.

Cells were resuspended and stained with one of the following mAb: LRTC1 (anti-LFA-1), R73 (anti-TCR) ED1 (macrophage-specific mAb), B36 (PEC/T cell negative) followed by addition both FITC-conjugated F(ab')$_2$ rat anti-mouse, and streptavidin Red-670 and were analyzed by flow cytometer. PEC-T cell doublets appearing as TCR-positive macrophages were excluded by gating and visual counting of cultures.

3.7 A possible function of the incorporation of TCR by macrophages

Because PEC in the presence of T cells appeared to be incorporating TCR, experiments were performed to determine whether this process affected their function. To discover macrophages bearing T cell surface proteins was a significant find in itself, but to pinpoint a function to it would be important. The first task was to determine if irradiated T cells could reactivate resting macrophages. Under normal conditions, T cells will reactivate resting macrophages by the production of cytokines after interaction with MHC plus antigen . Irradiated T cells did reactivate macrophages in the presence of GPMBP, evident by the production of nitric oxide (Fig. 14). The results indicate that the irr T cells are not playing an active role in the reactivation of macrophages.

In the experiments that followed, irradiated macrophages were cultured with and without irradiated T cells for 72 hours. This allowed time for the T cells to die off. Supernatants were withdrawn which allowed only adherent macrophages to be plated with and without T cells and GPMBP. There should have been no difference in the results from the original two cultures, but the data revealed that the macrophage / T cell culture, which no longer contained T cells (no IL-2 production), produced NO in the presence of GPMBP where the macrophage culture did not (Fig. 15). Remembering that the production of NO by macrophages is an antigen-specific function, this implies that macrophages are incorporating TCR from the T cells which allows the macrophages to respond to GPMBP, hence, producing NO.

3.8 NO production in EAE

The active sensitization of Lewis rats was employed to determine at what point of disease NO production was at its highest. Results have varied as to when this occurred. But the data indicated that induction of NO production by SPL was at its highest in rats with peak disease (Fig. 16). These results would suggest that NO plays a major role in the etiology of EAE.

3.9 Adoptive transfer studies suggest a more 'active' role for macrophages in EAE

When T cells are cultured with high densities of irradiated SPL and GPMBP, they are activated and display a large blastogenic morphology, high levels of IL-2 responsiveness, and readily transfer disease to recipient Lewis rats. When T cells are cultured with high densities of irrPEC and GPMBP, their morphology is very different, only a very few survive, having a small resting appearance and lower antigen responsiveness. Basically these cells look and act as if they are dying and unactivated, yet they still transfer disease (Table 3). This was rather unique because resting T cells cultured with macrophages and GPMBP do not transfer disease. This observation is suggestive that macrophages may actively contribute to EAE transfer in this experimental system.

Figure 14. Irradiated T cells can reactivate resting PEC as evidenced by NO production. PEC (5 x 10^5/ml) were cultured in cRPMI plus 20% M-CSF for 4 days. Adherent cells were plated with either irradiated or non-irradiated T cells (2.5 x 10^4/well), with and without LPS (10 μg/ml) and 2 μM GPMBP for 48 hours. **Nitric oxide production.** The production of nitric oxide, as measured by formation of nitrite, was determined using a microplate assay described in Materials and Methods. Supernatant (50 μl) was mixed with equal amounts of Griess reagent and read on an ELISA plate reader, represented on the Y axis as the change in optical density (ΔOD). Graphs were plotted as a function of the addition of GPMBP. These data are representative of two experiments.

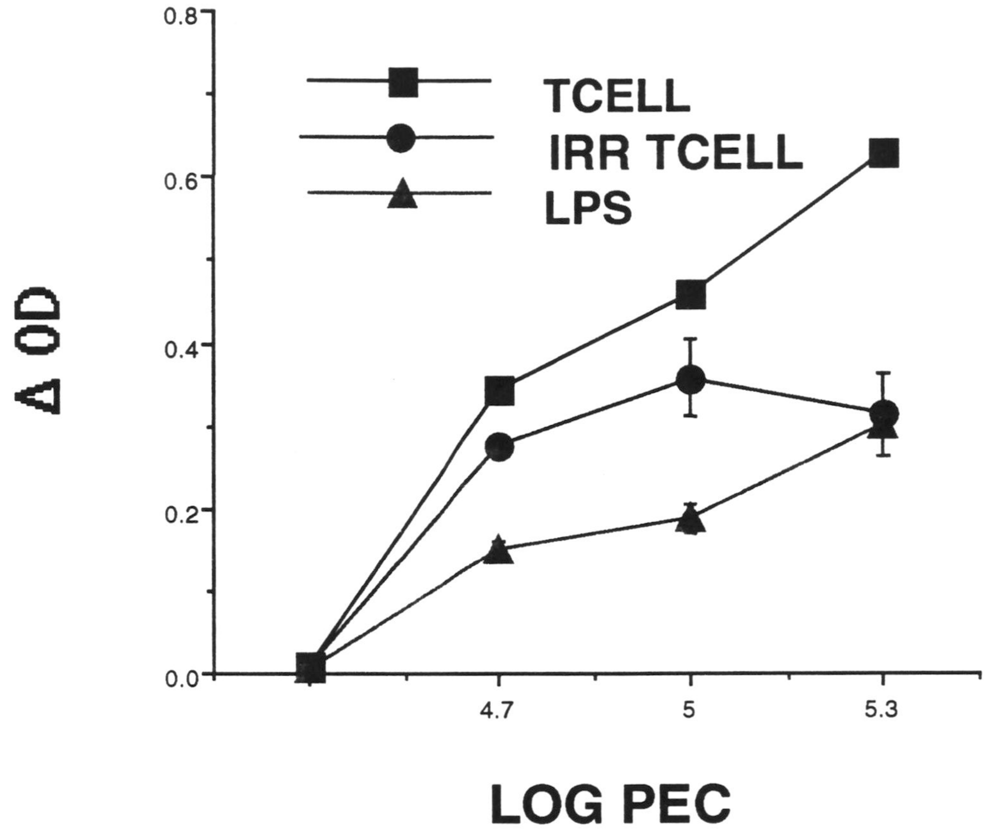
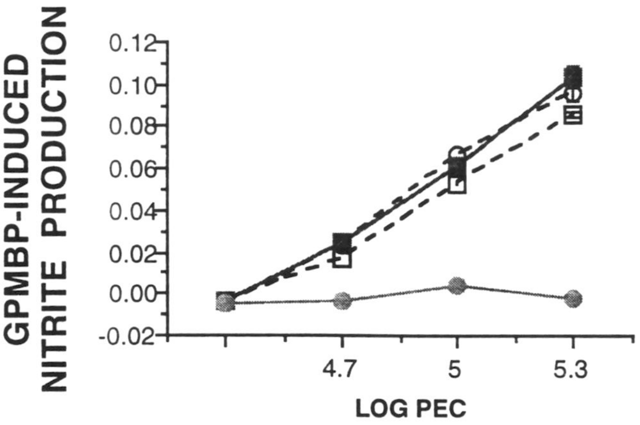
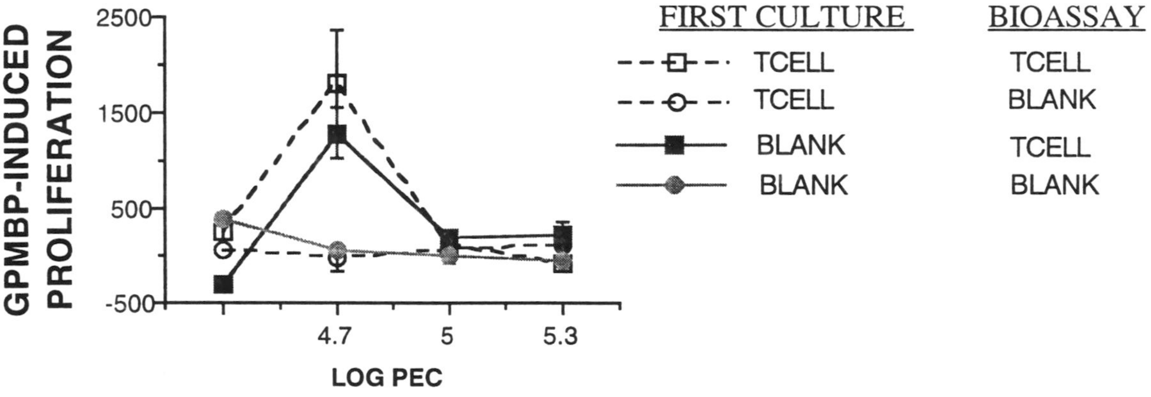

0.8
0.6
0.4
0.2
0.0
Δ OD
TCELL
IRR TCELL
LPS
4.7
5
5.3
LOG PEC

Figure 15. TCR-positive macrophages acquire antigen-specificity. Irradiated PEC (20 x 10^6/dish) were cultured with 3.2 mM AG in cRPMI with and without irradiated T cells for 72 hours (1° culture). At the end of the incubation, cells were plated with and without T cells (2.5 x 10^4/well) and 2 μM GPMBP for 48 hours (2° culture). **Measurement of proliferation and IL-2 production.** Supernatants were transferred to replicate plates, and T cells were harvested and counted to measure [^{3}H]thymidine incorporation. To determine IL-2 activity, CTLLs (5 x 10^4/ 50 μl/ well) were cultured with supernatants for 24 hours. During the last 4 hours of culture, 20 μl of MTS were added to each well to measure viability and growth. **Nitric oxide production.** The production of nitric oxide, as measured by formation of nitrite, was determined using a microplate assay described in Materials and Methods. Supernatant (50 μl) was mixed with equal amounts of Griess reagent and read on an ELISA plate reader. Graphs were plotted as a function of the addition of GPMBP. These data are representative of two experiments.


GPMBP-INDUCED
NITRITE PRODUCTION
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
4.7
5
5.3
LOG PEC


GPMBP-INDUCED
PROLIFERATION
2500
1500
500
-500
4.7
5
5.3
LOG PEC
FIRST CULTURE
BIOASSAY
TCELL
TCELL
TCELL
BLANK
BLANK
TCELL
BLANK
BLANK

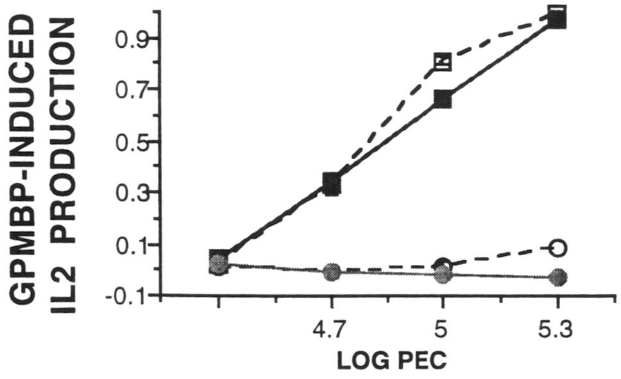

GPMBP-INDUCED
IL2 PRODUCTION
0.9
0.7
0.5
0.3
0.1
-0.1
4.7
5
5.3
LOG PEC

Table 3

Comparative APC activities of irrSPL and irrPEC for adoptive transfer of EAE

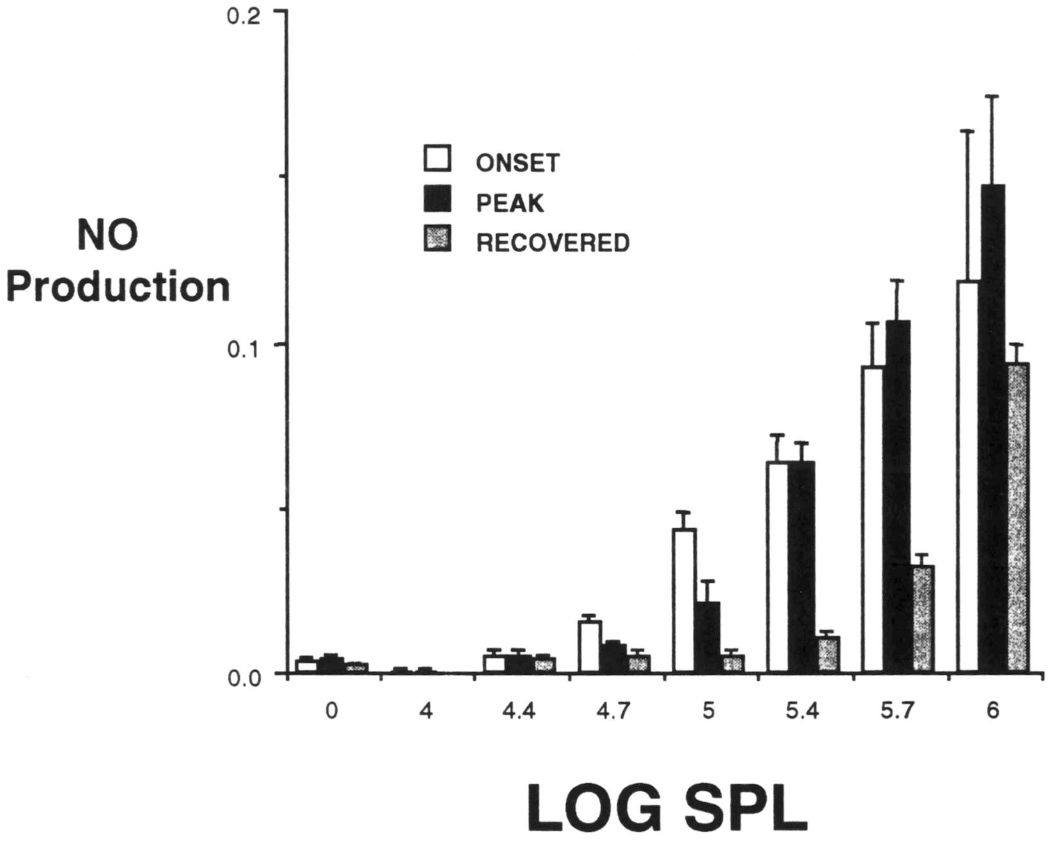
Irradiation of T cells	Type of APC added to culture[a]	APC/T cell ratio[b]	Addition of GPMBP	Incidence of EAE (mean maximal intensity)[c]
-	IrrSPL	10	+	(0.5)
-	IrrPEC	10	+	(0.3)
+	IrrPEC	10	+	(0.0)
-	IrrPEC	10	-	(0.0)

[a] PEC were elicited by i.p. injection of 200 μg *C. parvum* in 5 ml Hank's buffered saline solution and were recovered after 2 days from Lewis rats.

[b] MBP-specific T cells (2.5×10^6/ petri dish) were cultured with 25×10^6 irrSPL or irrPEC in the presence or absence of 2 μM GPMBP. After 2 days of culture, cells were extensively washed and injected i.v. into Lewis rat recipients.

[c] The following scale was used to assign intensity of EAE; paralysis in the distal tail, 0.25, limp tail, 0.5, ataxia, 1.0, hindleg paresis, 2.0, full hindleg paralysis, 3.0. The mean maximal intensity was the average maximum score among afflicted rats within a group.

FIGURE 16. Nitric oxide production is optimum at peak disease. Three Lewis rats were challenged with 50 µg GPMBP/ 0.1 ml CFA in emulsion. SPL were harvested from rats upon onset of EAE, peak disease and recovery. Assays were set up of each with 1 and 3 mM AG, with and without 2 µM GPMBP and 2.5 µg/ml Con A for 48 hours. **Nitric oxide production.** The production of nitric oxide, as measured by formation of nitrite, was determined using a microplate assay described in Materials and Methods. Supernatant (50 µl) was mixed with equal amounts of Griess reagent and read on an ELISA plate reader. Graphs were plotted as a function of the addition of GPMBP. These data are representative of two experiments.


0.2
0.1
0.0
NO Production
ONSET
PEAK
RECOVERED
0
4
4.4
4.7
5
5.4
5.7
6
LOG SPL

Chapter 4

DISCUSSION

4.1 SPL and PEC react differently in the presence of T-helper cells

Splenocytes are highly efficient antigen presenting cells. When cultured in the presence of GPMBP and GPMBP-specific $CD4^+$ T cells, they present the antigen to the T cells which undergo differentiation and secretion of cytokines (IL-2). This is the typical APC / T cell interaction leading to the production of a sustained population of proliferating T cells. On the other hand, when PEC from Lewis rats were used in the place of SPL and cultured in a similar fashion, IL-2 was still produced but T cell proliferation decreased in a dose dependent manner. Our data show that the peritoneal macrophages were presenting antigen to the T cells which in turn produced IL-2, but the macrophages were then killing the T cells by an unknown mechanism. The novel interaction between APC / T cell lead to the further investigation of the inhibitory phenomenon.

4.2 T cells are undergoing cell death mediated by macrophages

Peritoneal macrophages were determined to be inhibiting the proliferation of T cells by the active form of cell death. Initial research had failed to pinpoint large populations of dead cells. Definitive proof was found, however, using the sub-diploid flow cytometric method to identify sub-diploid (dead) cells. This discrepancy was ultimately due to the fact that the macrophages were promptly phagocytosing the dead T cells. So there was only a small window of opportunity when there were dead T cells present to be identified. In earlier studies, apoptosis (programmed cell death) had been shown to be responsible for the deletion of autoreactive T cells *in vivo* during the normal development of tolerance in EAE (Pender et al., 1991). Deletion of myelin / oligodendrocyte-specific lymphocytes in the CNS in EAE could explain both the subsidence of inflammation and the acquisition of tolerance. The present system was postulated as an *in vitro* example of peripheral T cell tolerance where macrophages could selectively delete autoreactive T cells in the periphery that had escaped clonal deletion in the thymus (Ichikawa et al. 1996, Munn et al., 1996, Nossal 1994). Shortly afterward, Munn and co-workers published a similar hypothesis using activated T cells in the presence of M-CSF-derived macrophages (Munn et al., 1996). This idea was further supported when alveolar macrophages from rodents were shown to selectively inhibit T cell proliferation by NO mediated apoptosis but permitted T cell activation and secretion of IL-2 (Upham et al., 1984). It is clear from published literature that one mechanism by which rodent macrophages inhibit T cell proliferation involves activation of NOS and production of reactive nitrogen intermediates. The present PEC / T cell model is a good example of this interaction.

4.3 Nitric oxide is a mediator of T cell death

A number of mechanisms have been found to induce T cell death *in vitro*. T cells can be killed when glucocorticosteroids are added (Cohen, 1993), when submitted to high doses of irradiation, or even by a strong antigenic challenge. But the mediator of T cell death in this system was determined to be nitric oxide. Early experiments showed that NO inhibitors did inhibit the T cell death, but it was not until the use of the Griess reaction that it could confirmed that the production of toxic levels of NO by macrophages was the cause of T cell death.

Nitric oxide had been suspected for years to be involved in the regulation of vascular tone and neurotransmission, and in the killing of microbes and tumor cells. But it was not until the late 80's and early 90's that research suggested a role in autoimmune disease. Macrophages found around lesions in EAE had been proven to produce NO (Albina et al., 1993, Ding et al., 1988, Kitajima et al., 1994). Nitric oxide was also implicated in the destruction of T cells, oligodendrocytes, and tissue damage (Okuda et al., 1995, Wei et al., 1995). Around the time this research began, NO had become a hot topic. But there seems to be two schools of thought when it comes to NO's involvement in EAE. The first is that NO is a major contributor to the manifestation of the disease (Hewett et al., 1996, Rivero et al., 1997, Zhao et al., 1996). In other experiments, nitrogen and oxygen free radicals have been found in the inflammatory cells isolated from the CNS of rats with EAE (Cross et al., 1996). Inducible NOS mRNA has been detected in the brains of rats with EAE (Cross et al., 1996, Hooper et al., 1997). The presence of NO complexed to iron-sulfur proteins has been demonstrated in the spinal cords of mice with EAE (Cross et al., 1996, Ruuls et al., 1996). High expression of iNOS was found to coincide with severity of EAE, and iNOS inhibitors were shown to inhibit EAE (Cross et al., 1996, Hooper et al., 1997, Rivero et al., 1997, Zhao et al., 1996).

The opposing view is that NO actually aids in the clearing of disease (Bauer et al., 1995, Pender et al., 1991, Ruuls et al., 1996, Tabi et al., 1995). NO has been shown to prevent the over expansion of Th1 cells implicated in many autoimmune diseases (Wei et al., 1995). These same dead cells have been found in the lesions in EAE (Bauer et al., 1995). Research has also implicated iNOS inhibitors in aggravating EAE (Ruuls et al., 1996, Zielasek et al., 1995), and revealed the down-regulation of MBP-specific T cells leading to the spontaneous recovery and the development of tolerance (Tabi et al., 1995). The present research demonstrates that NO kills autoreactive T cells, suggesting an immune-suppressive role for NO. The actual situation is probably more like a feedback inhibition, where levels of NO regulate homeostasis. Low concentrations of NO carry out demyelination and oligodendrocyte damage and disease worsens. As more macrophages are activated and the concentration of NO increases, autoreactive T cells and macrophages are killed, clearing disease. This same thinking could be applied to Multiple Sclerosis. Until recently it was thought that human macrophages did not produce NO, but recent evidence has shown this to be untrue. Several human macrophage lines have been stimulated to produce NO *in vitro* and *in vivo*. (DeVera et al., 1996, Hooper et al., 1997, Vouldoukis et al., 1995).

4.4 Antigen specificity of NO-mediated T cell death

The fact that the pathogenic mechanisms in EAE are highly specific and that macrophages have been identified as the primary end-stage effector cells in EAE argue that macrophages have antigen-specific functions. Though macrophages have always been believed to be nonspecific, when they are cultured with GPMBP-specific T cell lines without antigen, the T cells are not killed, but when they are cultured with T cells and GPMBP, the T cells are killed. In the absence of injury or infection, the majority of macrophages present only host-derived proteins. Applying the same logic that underlies intrathymic deletion, T cells that respond to antigen in this context should be considered autoreactive, and therefore candidates for deletion (Munn et al., 1996). These findings back this theory, whereby the production of NO and subsequent killing of MBP-specific T cells by macrophages was shown to be antigen-specific in nature. Results of these experiments showed that IL-2 had an adverse effect on the antigen specificity of NO production. In early experiments PEC were rested in CM (IL-2-conditioned medium) to aid in propagation. When these rested PEC were plated with T cells with and without GPMBP, there was no difference in NO production. Thus NO production seemed to be antigen-specific. When the results of PEC cultured in CM were compared to regular cRPMI treatment, it was realized that the IL-2 removed the antigen-specificity of NO production found in the cRPMI culture (Fig. 10). As of yet no other researchers have reported these findings. Several studies have tried without success to demonstrate that NO regulates IL-2 production (Marcinkiewicz and Chain, 1993, Marcinkiewicz et al., 1996), but evidence does support its regulation of IL-3 and IL-4 (Taylor-Robinson et al., 1994). The present research seems to suggest that IL-2 plays a role in NO regulation, perhaps by switching the enzyme responsible for NO production or by blocking one enzyme (iNOS) leaving only the other (cNOS) to produce NO.

4.5 The acquisition of T cell receptor by macrophages

The very idea that TCR or other T cell-surface proteins would be expressed on macrophages was quite unique. Yet early experiments revealed that, after culture with irradiated T cells, peritoneal macrophages were found to stain positive for CD2, CD5, and TCR, all of which are not known to exist on macrophages. If such proteins are found on macrophages after culture with T cells, the possibility remains that macrophages did not incorporate these proteins pre-made from T cells but rather synthesized these proteins via unknown regulatory strategies. This possibility does not however exist for the TCR. The antigen receptor of T cells is a heterodimer composed of alpha / beta chains which are the primary recognition domain, associated with a complex of 3 dimers (CD3) that serve as the primary signal transduction complex. The alpha/beta heterodimer and CD3 must coexist in the membrane due to intermolecular salt bridges within the hydrophobic membrane spanning domains. Both the alpha and beta chains of the TCR are encoded by uniquely rearranged DNA gene segments during cellular maturation. Thus, DNA encoding the TCR alpha and beta chains are unique to each T cell clone and are not reproduced in different T cell clones or in different somatic cells. If macrophages express the T cell receptor, then this molecular complex was somehow captured from a T cell and recycled to the cell surface.

Several facts should be considered when analyzing this data. First, PEC are comprised of 30-50% T cells along with around 30% macrophages. Thus, cultures with irr PEC alone actually contain some T cells which die due to irradiation. These residual T cells may be responsible for low levels of R73 binding to these macrophages. Much higher levels of R73 binding are found in PEC cultured with MBP-specific T cells. Second, macrophages cultivated with T cells and antigen were much healthier than macrophages cultured alone. This effect is due to the T cell secretion of IL-2. Reduced viability of macrophages accounts for the higher degree of nonspecific binding since dead cells bind secondary fluorochromes nonspecifically. Third, activated T cells express lower levels of TCR than resting T cells. This observation has been documented in several other laboratories and appears to be due to the activation-dependent shedding of TCR during blastogenesis (Dustin et al., 1996, Rabinowitz et al., 1995). Fourth, at least two other experiments have provided preliminary data supporting the observation that macrophages can acquire T cell markers. Previous studies have provided definitive evidence that under select experimental conditions, macrophage-derived vesicles are captured by T cells. Macrophage vesicular proteins are transferred to the T cell surface presumably by a fusion event, where these proteins confer new T cell functions. The premise is that if these cell-surface proteins can go in one direction (from macrophage to T cell) perhaps they can go in the other (from T cell to macrophage). This research, though very thorough, leaves some question as to whether this phenomenon is actually occurring, but studies are under way to verify this premise.

4.6 A function for the binding of T cell receptor

Preliminary research found that irradiated T cells (functionally dead) were able to reactivate resting peritoneal macrophages determined by their ability to produce NO. Follow-up experiments revealed macrophages cultured with T cells then purified were reactivated by the addition of only GPMBP. To make sure no T cells were present in the bioassay, original T cells were irradiated which killed them, and supernatants from dishes were decanted so that only adherent macrophages would be present. This plus the fact that no IL-2 was produced would imply that there were no residual T cells. These results indicate that the incorporation of TCR by PEC imparts macrophages with antigen-specific functionality. Researchers have confirmed that macrophages were the end-stage effector cells in EAE, but could not understand how they could perform such an antigen-specific task. This research suggests that macrophages in the presence of autoreactive T cells in the parenchyma are acquiring TCR. This newly acquired TCR used in conjunction with their own MHC allows them to specifically target and destroy CNS myelin.

The exact mechanism in which macrophages could recover TCR from T cells is not known, but there are several possibilities. One theory is that PEC could be acquiring TCR through contact with TCR bearing vesicles from apoptosing T cells, or by recycling TCR from engulfed cells. Immunogold labeling has revealed the presence of T cell surface membrane molecules (TCR) and MHC class I molecules on the membranes of CTL granules which might ensure unidirectional delivery of lethal compounds to target cells (Peters et al., 1990). Another belief is that after disengagement, molecules which are pulled from the T cell membrand may remain attached to the PEC (Dustin et al., 1996, Rabinowitz et al., 1995). Downregulation of TCR is seen after disengagement of T cell hybridomas to planar bilayers containing MHC class II plus peptide (Dustin et al., 1996). Conclusive evidence of TCR incorporation by macrophages could be gained by immunoprecipitating TCR on cultures which had been sorted and propagated to ensure cellular homogenicity. Confirmation of this theory could have broad implications for the role macrophages play in both autoimmune pathogenesis and normal cell-mediated responses.

Chapter 5

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