Neural, endocrine and immune interactions at the level of circulating leucocytes in multiple sclerosis

A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Science, University of London.

by

Ioannis Antonios Zoukos

Department of Neurochemistry, Institute of Neurology, London.
ABSTRACT

Interactions between the neural, endocrine and immune systems at the level of peripheral blood mononuclear cells (PBMCs) have been investigated in multiple sclerosis (MS). An increased density of β-adrenoceptors was demonstrated on PBMCs in patients with progressive or relapsing-remitting disease. The same observation was made in patients with chronic rheumatoid arthritis (RA), but not in myasthenia gravis (MG). The affinity of the receptors was within the normal range and there was a positive correlation between density and function as determined by intracellular cAMP production after stimulation. In vitro, functional increased expression of β-adrenoceptors was observed when PBMCs from normal controls were cultured in the presence of the soluble mediators interleukin-1 (IL-1) and hydrocortisone, whereas the already up-regulated receptors on PBMCs from MS patients remained unchanged. Further support for the role of those mediators in β-adrenoceptor expression was given by the observation that plasma cortisol levels were high in patients during relapse.

The link between high density of β-adrenoceptors and inflammation was further elucidated in a serial monthly assessment of relapsing-remitting MS patients, where it was demonstrated that the increase in β-adrenoceptors on
PBMCs was correlated with expression of high affinity IL-2 receptors (IL-2Rs) and disease activity as determined by clinical and magnetic resonance imaging (MRI). Furthermore, plasma levels of cytokines such as tumour necrosis factor alpha (TNFα) and interleukin-1 beta (IL1β), appeared to be increased during the active phase of the disease.

The hypothesis that β-adrenoceptor increased expression may be due to autonomic dysfunction was tested in a comparative study of MS patients, normal subjects and two models of autonomic dysfunction, pure autonomic failure (PAF) and multiple system atrophy (MSA). Only in PAF did β-adrenoceptor up-regulation correlate with low plasma levels of noradrenaline (NA) and adrenaline (Ad). Central autonomic function and neurohormonal responses to the centrally acting sympatholytic agent, clonidine, were studied. Although the haemodynamic and neurohormonal responses to clonidine, suggest a central autonomic dysfunction in progressive MS patients, the disparity between MS and PAF patients, when considering their plasma levels of NA and Ad, indicate that high density of β-adrenoceptors on PBMCs in MS is more likely to be due to the inflammatory process than autonomic dysfunction.

A link between β-adrenoceptor increased expression on PBMCs and recovery has been suggested by in vitro studies in which β-agonist stimulation was found to reduce the IL-
2βR density and suppress cell proliferation following mitogenic stimulation. The therapeutic importance of β-adrenoceptor up-regulation in MS remains to be tested by appropriate trials using either β-agonists or agents activating the second messenger system, adenylate cyclase, in lymphocytes.
CONTENTS

Title Page 1
Abstract 2
Contents 5
Figures 12
Tables 15
Abbreviations 17
Acknowledgements 20

CHAPTER 1
INTRODUCTION

1.1 The Central Nervous System as an Immunologically privileged site 23
1.2 The effects of the Nervous System upon the Immune System 25
1.2.1 Neuroimmunomodulation 27
1.2.2 Neuroendocrinology 28
1.2.3 Neural, Endocrine and Immune interactions 30
1.2.3 Neuroregulators and receptors common to immunocompetent and neuroendocrine cells 34
1.3 The effects of the Immune System upon the Nervous System 34
1.3.1 Neuroimmunology 34
1.4 Effects of Inflammation on Neuroendocrine Function 40
1.4.1 Activation of the pituitary and adrenal gland by inflammation 40
1.5 Neural and endocrine responses in EAE 43
1.6 Neural, endocrine and immune interactions in MS 45
1.6.1 The hypothalamic-pituitary-adrenal (HPA) axis and ACTH in MS 46
1.6.2 Sex hormones and MS 48
1.6.3 The Sympathetic Nervous System (SNS) in MS 49
1.7 Aims of this Thesis 51
CHAPTER 2

DISTRIBUTION OF $\beta$-ADRENOCEPTORS AND INTERLEUKIN 2 RECEPTORS (IL-2Rs) ON RESTING AND ACTIVATED PERIPHERAL BLOOD MONONUCLEAR CELLS: IN VITRO STUDIES

2.1 $\beta$-adrenoceptors 54
2.1.1 $\beta$-adrenoceptor subtypes 54
2.1.2 Localization of $\beta$-adrenoceptors 57
2.1.3 Measurement of $\beta$-adrenoceptors in mononuclear leukocytes 58
2.1.4 Distribution of $\beta$-adrenoceptors in lymphocyte subsets and monocytes 59
2.1.5 Methods 60
2.1.6 $\beta$-adrenoceptor binding on peripheral blood mononuclear cells using $(-)\text{I}^{125}$ cyanopindolol (ICYP) 60
2.1.7 Isolation of peripheral blood mononuclear cells (PBMCs) 61
2.1.8 Binding characteristic of $(-)\text{I}^{125}$ cyanopindolol (ICYP to intact PBMCs) 62
2.1.9 Kinetics for $\text{I}^{125}$-CYP binding 63
2.1.10 Competition for $\text{I}^{125}$-CYP binding with $(-)$ propranolol, atenolol, ICI 118551 and phentolamine 66
2.1.11 Binding characteristics of $(-)\text{I}^{125}$-CYP in concentrations above 300 pM 76
2.1.12 Results 77
2.1.13 Discussion 70
2.2 $\beta$-adrenoceptor second messenger system 72
2.2.1 G Proteins 72
2.2.2 Adenylate Cyclase 72
2.2.3 cAMP-dependent protein kinase 73
2.2.4 Adenosine 3',5'-Cyclic Monophosphate Assay 74
2.2.5 Measurements of intracellular cyclic AMP 75
2.2.6 Intracellular cAMP production after stimulation with $\beta$-adrenoceptor agonist, $(-)$-Isoproterenol 76
2.2.7 Intracellular production of cAMP after stimulation of PBMCs with Forskolin

2.2.8 Discussion

2.3 Expression of β-adrenoceptors on PBMCs before and after stimulation with steroids, interleukin-1 and Phytohemagglutinin (PHA)

2.3.1 The effect of steroids on PBMC β-adrenoceptors

2.3.2 The effect of interleukin-1 on PBMC β-adrenoceptors

2.3.3 The effect of mitogenic stimulation with PHA on PBMC β-adrenoceptors

2.3.4 Basal and isoproterenol-stimulated intracellular production of cyclic AMP in cultured PBMCs in the presence of hydrocortisone IL-1α or PHA

2.3.5 Statistical analysis

2.4 Interleukin-2 receptor (IL-2R)

2.4.1 Methods

2.4.2 High affinity IL-2 receptor (IL-2R) binding on peripheral blood mononuclear cells using 125I Interleukin-2

2.4.3 Kinetics for 125I IL-2 binding

2.4.4 Phytohemagglutinin (PHA)-induced lymphocyte proliferation

2.4.5 High affinity IL-2R on PBMCs stimulated with PHA

2.4.6 Results

2.4.7 Discussion

2.5 Regulation of proliferation rate and expression of IL-2Rs through β-adrenoceptor stimulation

2.5.1 Regulation of high affinity IL-2R expression on PBMCs by stimulating β-adrenoceptors

2.5.2 Proliferation rate of stimulated by PHA PBMCs in the presence of β-agonist Isoproterenol

2.5.3 Regulation of high affinity IL-2Rs on activated PBMCs by β-agonist or hydrocortisone
CHAPTER 3

EXPRESSION OF \(\beta\)-ADRENOCEPTORS AND IL-2 RECEPTORS ON
PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCs) IN MULTIPLE
SCLEROSIS

3.1 \(\beta\)-adrenoceptor densities on PBMCs in MS patients; a regulatory role for
cortisol and interleukins?

3.1.1 Methods

3.1.2 Subjects

3.1.3 Isolation of PBMCs and \(\beta\)-adrenoceptor assay

3.1.4 Plasma cortisol determination

3.1.5 Measurement of intracellular cAMP

3.1.6 Statistical analysis

3.1.7 Results

3.1.8 \(\beta\)-adrenoceptor density on PBMCs

3.1.9 Basal and isoproterenol-stimulated intracellular production of cAMP

3.1.10 Plasma levels of cortisol

3.1.11 The effect of hydrocortisone and interleukin-1 on PBMC \(\beta\)-adrenoceptors from normal subjects and secondary progressive MS patients

3.1.12 Discussion

3.2 Expression of high affinity IL-2R and \(\beta\)-adrenoceptors on PBMCs;
correlations with clinical and MRI activity in relapsing-remitting MS patients

3.2.1 Methods

3.2.2 Subjects

3.2.3 Magnetic Resonance Imaging (MRI)

3.2.4 \(\beta\)-Adrenergic receptor assay

3.2.5 Interleukin-2 receptor assay

3.2.6 IL-2R densities on PBMCs after stimulation with PHA in the presence and absence of isoproterenol
3.2.7 Phytohemaglutinin (PHA)-stimulated lymphocyte proliferation in the presence and absence of isoproterenol

3.2.8 Statistical analysis

3.2.9 Results

3.2.10 β-adrenoceptor densities on PBMCs

3.2.11 High affinity IL-2 receptor densities on PBMCs

3.2.12 IL-2R densities on PBMCs from NC and MS patients after stimulation with PHA in the presence or absence of isoproterenol

3.2.13 PHA-induced lymphocytic proliferation in the presence and absence of isoproterenol in NC and MS patients

3.2.14 IL-2R expression on PBMCs from MS patients during relapse before and after stimulation with isoproterenol

3.3 Circulating cytokines in MS patients

3.3.1 Methods

3.3.2 Levels of TNFα, IL-1α, IL-1β and IL-6

3.3.3 Cytokine serum levels

3.3.4 Cytokines after stimulation of whole blood with PHA

3.4 Discussion

CHAPTER 4

AUTONOMIC FUNCTION AND EXPRESSION OF β-ADRENOCEPTORS ON PBMCs IN MULTIPLE SCLEROSIS AND AUTONOMIC FAILURE

4.1 Autonomic Nervous System

4.1.1 Peripheral autonomic function

4.1.2 Central control of the autonomic nervous system

4.1.3 Diseases of the autonomic nervous system

4.1.4 β-adrenoceptor densities on PBMCs in autonomic failure patients

4.1.5 Autonomic function and expression of β-adrenoceptors on PBMCs in multiple sclerosis patients
4.2 Physiological assessment of autonomic function in progressive MS patients

4.2.1 Methods

4.2.2 Cardiovascular testing

4.2.3 Postural challenge

4.2.4 Valsalva manoeuvre (VM)

4.2.5 Pressor stimuli

4.2.6 Heart rate responses to respiratory change

4.2.7 Noninvasive quantification of superior mesenteric artery (SMA) blood flow during sympathoneural activation

4.2.8 Cardiac index (CI)

4.2.9 Forearm muscle blood flow (FBF)

4.2.10 Digital skin blood flow (DSBF)

4.2.11 Subjects

4.2.12 Statistical analysis

4.2.13 Results

4.2.14 Discussion

4.3 Assessment of central autonomic function in multiple sclerosis: cardiovascular and neurohormonal responses to clonidine

4.3.1 Methods and Subjects

4.3.2 Measurements of plasma levels of noradrenaline (NA) and adrenaline (Ad)

4.3.3 Growth hormone (GH) measurements

4.3.4 Statistical analysis

4.3.5 Results

4.3.6 Systemic blood pressure (BP) and heart rate (HR)

4.3.7 Regional hemodynamic measurements

4.3.8 Discussion
4.4 Expression of β-adrenoceptors on PBMCs before and after reduction of central sympathetic outflow by clonidine in normal subjects and in patients with Autonomic failure or MS

4.4.1 Methods and Subjects

4.4.2 The effects of clonidine on β-adrenoceptor density of PBMCs in vitro

4.4.3 Statistical analysis

4.4.4 Results

4.4.5 Effects of reduced central sympathetic outflow on PBMC β-adrenoceptor densities in normal subjects

4.4.6 PBMCs β-adrenoceptors before and after reduction of central sympathetic outflow in MSA, PAF, MS, and NS

4.4.7 Discussion

4.4.8 Conclusions

CHAPTER 5

DISCUSSION

REFERENCE LIST

APPENDIX
## FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Schematic representation of sympathetic nervous system-neuroendocrine-immune interactions.</td>
<td>39</td>
</tr>
<tr>
<td>2.1</td>
<td>Functional domains of the $\beta_2$-adrenoceptor.</td>
<td>56</td>
</tr>
<tr>
<td>2.2</td>
<td>Binding characteristics of $\beta$-adrenoceptors.</td>
<td>64</td>
</tr>
<tr>
<td>2.3</td>
<td>Kinetics for $^{125}$ICYP binding.</td>
<td>65</td>
</tr>
<tr>
<td>2.4</td>
<td>Competitive binding of $\beta$-adrenoceptors.</td>
<td>68</td>
</tr>
<tr>
<td>2.5</td>
<td>$^{125}$ICYP binding between 10 to 500 pM.</td>
<td>69</td>
</tr>
<tr>
<td>2.6</td>
<td>cAMP assay.</td>
<td>78</td>
</tr>
<tr>
<td>2.7</td>
<td>Intracellular cAMP production after stimulation with isoproterenol.</td>
<td>79</td>
</tr>
<tr>
<td>2.8</td>
<td>Intracellular cAMP production after stimulation with Forskolin.</td>
<td>80</td>
</tr>
<tr>
<td>2.9</td>
<td>$\beta$-adrenoceptors on PBMCs after stimulation with hydrocortisone.</td>
<td>84</td>
</tr>
<tr>
<td>2.10</td>
<td>Time course development of hydrocortisone effect upon $\beta$-adrenoceptor expression of PBMCs.</td>
<td>85</td>
</tr>
<tr>
<td>2.11</td>
<td>Effects of IL-$1\alpha$ upon $\beta$-adrenoceptor expression in PBMCs.</td>
<td>86</td>
</tr>
<tr>
<td>2.12</td>
<td>Time course of development of the IL-$1\alpha$ induced increase in $\beta$-adrenoceptor density.</td>
<td>87</td>
</tr>
<tr>
<td>2.13</td>
<td>Effects of PHA upon $\beta$-adrenoceptor expression in PBMCs.</td>
<td>88</td>
</tr>
<tr>
<td>2.14</td>
<td>Intracellular cAMP production in PBMCs stimulated by hydrocortisone, IL-$1\alpha$ and PHA.</td>
<td>89</td>
</tr>
<tr>
<td>2.15 (A)</td>
<td>Binding characteristics of $^{125}$I-IL-2.</td>
<td>92</td>
</tr>
<tr>
<td>2.15 (B)</td>
<td>Binding characteristics of $^{125}$I-IL-2.</td>
<td>93</td>
</tr>
<tr>
<td>2.16</td>
<td>Kinetics for $^{125}$I-IL-2 binding.</td>
<td>94</td>
</tr>
<tr>
<td>2.17</td>
<td>Proliferation rate after stimulation with PHA.</td>
<td>97</td>
</tr>
<tr>
<td>2.18</td>
<td>IL-2R expression after stimulation with PHA.</td>
<td>98</td>
</tr>
</tbody>
</table>
2.19 Binding characteristics for IL-2Rs after stimulation with PHA.
2.19 (A) Binding characteristics for IL-2Rs after stimulation with PHA.
2.20 IL-2 receptor expression on PBMCs after stimulation with PHA and isoproterenol.
2.21 Proliferation rate of PBMCs after stimulation with PHA and isoproterenol.
2.22 IL-2Rs on PBMCs after stimulation with hydrocortisone or isoproterenol.
3.1 Cortisol assay.
3.2 Intracellular cAMP production in PBMCs from MS patients.
3.3 β-adrenoceptors on PBMCs from MS patients after stimulation with hydrocortisone or interleukin-1α.
3.4 β-adrenoceptors and clinical status.
3.5 Binding characteristics for IL-2Rs on PBMCs from MS patients.
3.6 Correlation between β-adrenoceptor and IL-2R expression on PBMCs in MS.
3.7 IL-2Rs on PBMCs from MS patients in the presence of PHA and isoproterenol.
3.8 Proliferation rate of PHA and isoproterenol stimulated PBMCs from MS patients.
3.9 IL-2Rs in relapsed MS patients after stimulation with isoproterenol.
3.10 Cytokine plasma levels in MS patients.
3.11 Production of cytokines after stimulation with PHA in NC and MS patients.
4.1 Peripheral autonomic system.
4.2 Diagram of cardiovascular control mechanisms.
4.3 Valsalva Manoeuvre.
4.4 Blood pressure and heart rate responses to cutaneous cold.
4.5 Deep breathing, heart rate and blood pressure.
4.6 SMA Ultrasound Imaging.
4.7 SMABF changes to pressor stimuli in MS.
<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.8</td>
<td>HPLC chromatograms of catecholamines.</td>
<td>194</td>
</tr>
<tr>
<td>4.9</td>
<td>MABP after clonidine in NS, MSA and PAF.</td>
<td>196</td>
</tr>
<tr>
<td>4.10</td>
<td>MABP after clonidine in NC and MS patients.</td>
<td>199</td>
</tr>
<tr>
<td>4.11</td>
<td>SMABF and SMAVR after clonidine in NC and MSA and PAF.</td>
<td>201</td>
</tr>
<tr>
<td>4.12</td>
<td>SMABF and SMVR in NC and MS.</td>
<td>202</td>
</tr>
<tr>
<td>4.13</td>
<td>MABP and GH changes after clonidine in MS.</td>
<td>204</td>
</tr>
<tr>
<td>4.14</td>
<td>Time course changes in β-adrenoceptors after clonidine.</td>
<td>218</td>
</tr>
<tr>
<td>4.15</td>
<td>Scatchard plot analysis before and after clonidine.</td>
<td>219</td>
</tr>
<tr>
<td>4.16</td>
<td>Intracellular cAMP before and after clonidine.</td>
<td>220</td>
</tr>
<tr>
<td>4.17</td>
<td>PBMC β-adrenoceptors after stimulation with clonidine.</td>
<td>221</td>
</tr>
<tr>
<td>4.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>125IICYP binding before and after clonidine.</td>
<td>222</td>
</tr>
<tr>
<td>4.19</td>
<td>NA levels and β-adrenoceptors in NS, MSA, PAF and MS.</td>
<td>223</td>
</tr>
<tr>
<td>4.20</td>
<td>Intracellular cAMP in NS, MS and PAF.</td>
<td>224</td>
</tr>
</tbody>
</table>
### TABLES

<table>
<thead>
<tr>
<th>Table No.</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Immunoregulatory effects of several hormones and peptides.</td>
<td>32</td>
</tr>
<tr>
<td>1.2</td>
<td>Hormones and neuropeptides found in immunocompetent cells.</td>
<td>36</td>
</tr>
<tr>
<td>2.1</td>
<td>$\beta$-adrenoceptor densities and affinity ($K_p$) on PBMCs in normal subjects.</td>
<td>67</td>
</tr>
<tr>
<td>2.2</td>
<td>IL-2R expression on resting and PHA-stimulated PBMCs from normal subjects.</td>
<td>96</td>
</tr>
<tr>
<td>3.1</td>
<td>Clinical criteria for classification of patients with MS.</td>
<td>113</td>
</tr>
<tr>
<td>3.2</td>
<td>Peripheral blood mononuclear cell receptor and plasma cortisol in MS, normal controls and other diseases.</td>
<td>118</td>
</tr>
<tr>
<td>3.3</td>
<td>$\beta$-adrenoceptor and IL-2 receptor densities on PBMCs, clinical status, disability score and MRI findings in relapsing remitting MS patients.</td>
<td>138</td>
</tr>
<tr>
<td>3.4</td>
<td>$\beta$-adrenoceptor and IL-2 receptor densities on PBMCs, clinical status, disability score and MRI findings in benign MS patients.</td>
<td>140</td>
</tr>
<tr>
<td>3.5</td>
<td>Observations in which PBMCs $\beta$-adrenoceptors are above 2000 sites/cell and IL-2Rs above 100 in clinical relapse, remission with MRI activity or not.</td>
<td>142</td>
</tr>
<tr>
<td>4.1</td>
<td>Multiple sclerosis patients in whom autonomic function was tested.</td>
<td>180</td>
</tr>
<tr>
<td>4.2</td>
<td>Average SBP, DBP, MABP, HR and CI before and during MA, CC, ISE and tilt at 2 and 10 minutes in normal controls and MS patients.</td>
<td>183</td>
</tr>
<tr>
<td>4.3</td>
<td>SMABF, SMAVR, FBF and FVR before and during MA, CC, ISE and tilt at 2 and 10 minutes in NC and MS patients.</td>
<td>184</td>
</tr>
<tr>
<td>4.4</td>
<td>Changes in MABP, HR, CI before and after clonidine in control subjects and patients with MSA, PAF and MS.</td>
<td>200</td>
</tr>
<tr>
<td>4.5</td>
<td>Changes in FBF, SDBF and FT before and after clonidine in normal subjects and MSA, PAF and MS patients.</td>
<td>203</td>
</tr>
</tbody>
</table>
4.6 Plasma levels of NA, Ad and GH before and after clonidine in controls and patients with MSA, PAF and MS.

4.7 Changes in MAP, SBP, DBP, β-adrenoceptor density on PBMCs, plasma levels of NA, Ad, DA and GH before and after clonidine in normal subjects.

4.8 β-adrenoceptor densities on PBMCs before and after clonidine in normal subjects, MSA, PAF and MS patients.
<table>
<thead>
<tr>
<th>ABBREVIATIONS</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>Ad</td>
<td>adrenaline</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diposphate</td>
</tr>
<tr>
<td>AF</td>
<td>autonomic failure</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>β-ADR</td>
<td>β-Adrenoceptors</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BP</td>
<td>blood pressure</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>C</td>
<td>catalytic</td>
</tr>
<tr>
<td>CC</td>
<td>cutaneous cold</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CGRP</td>
<td>calcitonin gene-related peptide</td>
</tr>
<tr>
<td>CI</td>
<td>cardiac index</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CPM</td>
<td>count per minute</td>
</tr>
<tr>
<td>CRF</td>
<td>corticotropin releasing factor</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>DB</td>
<td>deep breathing</td>
</tr>
<tr>
<td>DBP</td>
<td>diastolic blood pressure</td>
</tr>
<tr>
<td>DHA</td>
<td>dihydroalprenolol</td>
</tr>
<tr>
<td>DSBF</td>
<td>digital skin blood flow</td>
</tr>
<tr>
<td>DST</td>
<td>dexamethasone suppression test</td>
</tr>
<tr>
<td>E</td>
<td>enhanced</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental encephalomyelitis</td>
</tr>
<tr>
<td>EDSS</td>
<td>expanded disability status scale</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERR</td>
<td>early relapsing remitting</td>
</tr>
<tr>
<td>F/344</td>
<td>Fischer rat</td>
</tr>
<tr>
<td>FBF</td>
<td>forearm blood flow</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>Fig</td>
<td>figure</td>
</tr>
<tr>
<td>FVR</td>
<td>forearm vascular resistance</td>
</tr>
<tr>
<td>FT</td>
<td>finger Temperature</td>
</tr>
<tr>
<td>Gd-DTPA</td>
<td>gadolinium diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>GHRH</td>
<td>growth hormone releasing hormone</td>
</tr>
<tr>
<td>Gi</td>
<td>G protein inhibitor</td>
</tr>
<tr>
<td>Gs</td>
<td>G protein stimulator</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HPA</td>
<td>hypophysis pituitary adrenal axis</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>ICYP</td>
<td>iodocyanopindolol</td>
</tr>
<tr>
<td>i.c.v</td>
<td>intracerebroventricular</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon eg interferon-γ, or β</td>
</tr>
<tr>
<td>IHYP</td>
<td>iodohydroxybenzylpindolol</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin eg IL-1, IL-2, IL-6</td>
</tr>
<tr>
<td>IL-2R</td>
<td>interleukin-2 receptor</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneally</td>
</tr>
<tr>
<td>ISE</td>
<td>isometric exercise</td>
</tr>
<tr>
<td>IV</td>
<td>intravenous</td>
</tr>
<tr>
<td>LEW</td>
<td>Lewis rat</td>
</tr>
<tr>
<td>MA</td>
<td>mental arithmetic</td>
</tr>
<tr>
<td>MABP</td>
<td>mean arterial blood pressure</td>
</tr>
<tr>
<td>MG</td>
<td>myasthenia gravis</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>MSA</td>
<td>multiple system atrophy</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
</tbody>
</table>
NA
noradrenaline

NE
non-enhanced

NK
natural killer

PAF
pure autonomic failure

PBMCs
peripheral blood mononuclear cells

PBS
phosphate-buffered saline

PD
Parkinson's disease

PGE
prostaglandin E

PHA
phytohemaglutinin

PKA
protein kinase A

PKC
protein kinase C

POMC
propiomelanocortin

PVN
paraventricular nucleus

PWM
Pokeweed mitogen

RA
rheumatoid arthritis

RE
remission

RL
relapse

R
regulatory

SBP
systolic blood pressure

SDBF
skin digital blood flow

SIF
small intensely fluorescent

SMA
superior mesenteric artery

SMABF
superior mesenteric artery blood flow

SMAVR
superior mesenteric artery vascular resistance

SNS
sympathetic nervous system

SRBC
sheep red blood cells

TAV
time average velocity

TcR
T cell receptor

VIP
vasoactive intestine peptide

VM
Valsalva manoeuvre

VP
vasopressin

VR
vasopressin receptor
ACKNOWLEDGEMENTS

I would like to thank Professor M.L. Cuzner, my supervisor, and Dr J.P. Leonard for their guidance and encouragement throughout this study. I am grateful to Professor W.I. McDonald, Dr A. Thompson and Dr D. Kidd who kindly allowed me to study their patients and provided the relevant MRI information. I am also grateful to Professor C.J. Mathias and Dr T.N. Thomaides and Mr D. Pavitt for their collaboration in the autonomic function studies. I am indebted to Dr J. Loughlin for careful review of the early draft of the manuscript.

I would like also to acknowledge the support I received throughout my studies from Professor S. Balogiannis, my supervisor from the Greek State Scholarships Foundation. This work was funded by the Greek State Scholarships Foundation and the Multiple Sclerosis Society of Great Britain.

Dedicated to my parents
"Τη μεν τεχνη μακρα, ο δε βιος βραχυς "

Ηπποκράτης

"Art is infinite, but life is finite"

Hippocrates
CHAPTER 1

INTRODUCTION

The long-held view that homeostatic mechanisms are integrated by the nervous and endocrine systems has recently been expanded to include interactions with the immune system. Immune responses alter neural and endocrine function and, in turn, neural and endocrine activity modifies immunological function (Ader et al., 1991). Many regulatory peptides and their receptors previously thought to be limited to the brain or the immune system are now known to be expressed by both. Findings that link immune and neuroendocrine function provide explanations for the response of the pituitary and adrenal glands to infection and inflammation and the alterations in pituitary-thyroid and pituitary-gonadal function that occur in patients with non-endocrine disease. These findings may also explain how emotional state or response to stress can modify a person's capacity to cope with infection and influence the course of autoimmune disease.

These developments not only have heightened interest in neuroimmunology, which is the study of immune reactions involving the nervous system, but also have led to the coining of several new terms. "Neuroimmunomodulation" refers to the influence of the nervous system on the immune
response, "Psychoneuroimmunology" to the study of the effects of psychological status on immune function, and "Neuroendocrinoimmunology" to the study of neuroendocrine influences on the function of immunocompetent cells and the way these cells can influence neural function and endocrine activity.

1.1. The Central Nervous System as an Immunologically privileged site.

The central nervous system (CNS) is an immunologically privileged site in comparison with most other organs since the existence of the blood brain barrier (BBB) seals off direct interaction with immunocompetent cells. The BBB restricts the free passage of large molecules such as immunoglobulins as well as lymphocytes and other cells from circulation into the CNS whilst allowing active transport of certain molecules (Barker and Billingham, 1977; Griffin et al., 1984). The BBB, formed by tight junctions between the endothelial cells of the blood vessels in addition to a basement membrane and the astrocytic end-feet processes (Reese and Karnovsky, 1967), is however 'leaky' at the circumventricular organs allowing entry of plasma proteins into the extracellular space. There is also a low level of lymphocyte trafficking across the BBB and a small number of these cells are found throughout the normal CNS (Calder et al., 1989), while under certain conditions
immunocopotent cells may reach the brain and start an immunological reaction (Wekerle et al. 1986, Hickey 1990). While MHC class II antigen expression in the CNS of rodents is negligible (Vassand Lassman 1990), a constitutive expression of MHC molecules in human CNS has been reported (Hayes et al., 1987). However, when a general comparison between the immune and nervous system is made, it is intriguing to identify similarities in their functions. The nervous system is responsible for the perception and integration of information from the external and internal environment and the formation of appropriate responses. In order to achieve this, specialized centres for the internal homeostasis have been developed such as the hypothalamus, while for the interaction with the external environment, higher cognitive functions are essential and they are organized in cortical and subcortical structures of the brain. On the other hand, the functional unit of the lymphoid tissue, the leucocyte, acquires specific functions which can effectively protect the organism from external invaders. These cells are responsible for the self and non-self recognition and therefore for the initiation of an immune response. While a philosophical approach can easily identify those similarities, from the scientific point of view it is more interesting to discover means of intercommunication of the two systems which may subserve more effectively the maintenance of the organism's well being. The CNS possesses a sophisticated network through which it exchanges information with the immune system, and neural cells seem to
be able to function as immunocompetent cells under specific conditions. Such is supported for example by a number of observations related to the function of the microglia (embryologically and functionally related to macrophages) the existence of immune receptors for immune mediators such as interleukin 1 and glucocorticoids in various sites of the brain and the ability for local production of cytokines and expression of MHC within the CNS.

1.2. The effects of the nervous system upon the immune system.

1.2.1. Neuroimmunomodulation

Neural influences can modulate the immune response. In experiments in animals, Pavlovian conditioning induces immunosuppression (Ader et al., 1991), and lesions of the anterior hypothalamus modify asthma (Mrazek et al., 1991) and reduce humoral and cellular responses to foreign antigens (Felten et al., 1991). The susceptibility of Lewis rats (LEW) to experimental encephalomyelitis (EAE) or induced arthritis relative to disease resistant Fischer rats (F/344) is attributed to a hyporesponsive hypothalamus-pituitary-adrenal-axis (HPA) rather than immunological differences as the strains are histocompatible (Mason et al., 1990). Compromised hypothalamic pituitary adrenal (HPA) axis activity in the LEW rat is manifested in a reduction of inflammatory mediator-stimulated corticosterone and
adrenocorticotropic hormone (ACTH) secretion compared with F/344 rats (Sternberg et al., 1989). However, these appear to be the sequelae of a primary defect at the level of the hypothalamic corticotropin releasing factor (CRF) 41 neuron. Both CRF synthesis and secretion following a variety of stimuli are attenuated in the LEW rat relative to the F/344 rat, possibly as a consequence of a disrupted signal transduction mechanism in the CRF neuron (Calogero et al., 1992, Sternberg et al., 1989). Experimental destruction of the sensory innervation of joints (which includes neurons containing substance P, other tachykinins, and somatostatin) reduces the intensity of inflammatory arthritis (Kidd et al., 1989), indicating that locally released peptides or neurotransmitters can modify the inflammatory process. Intracerebral injections of bacterial endotoxin (Escherichia Coli lipopolysaccharide) or IL-1 inhibit the function of circulating B lymphocytes and macrophages and lead to the appearance of IL-6 in the circulation (De Simoni et al., 1990). Intracerebral injection of human immunodeficiency virus (HIV) glycoprotein (HIV-gp 120) decreases the function of peripheral-blood lymphocytes and macrophages (Sundar et al., 1991), suggesting that active Acquired Immunodeficiency syndrome (AIDS) encephalopathy may exert deleterious effects on the already compromised peripheral immune system. After head injury, several inflammatory cytokines including IL-6 appear in the circulation (McClain et al., 1991).

Changes in systemic immune function following
manipulations of the central nervous system suggest therefore that immunocompetence can be regulated by the brain. A relationship between brain and immune function is supported by the interesting observation that men and boys with dyslexia and left-handedness have a higher incidence of autoimmune disease (Geschwind et al., 1982).

1.2.2. Neuroendocrinology.

The nervous system can modulate the endocrine system in several ways (MacLean et al., 1981). Each of the anterior pituitary hormones is under the neuroendocrine control of the hypothalamus, and their secretion can be influenced by suprahypothalamic stimuli such as environmental signals, sleep rhythms, and physical and emotional stress. Furthermore, each of these hormones has either a direct or an indirect effect on the immune response through the secretions of its respective target gland. The most convincing example of neuroimmunomodulation by the hypothalamic-pituitary system is the activation of adrenocortical secretion. ACTH is released from the pituitary under the influence of CRF chiefly derived from neurons of the paraventricular nucleus (PVN) of the hypothalamus. ACTH causes release of glucocorticoids, potent immunosuppressive agents, from the adrenal cortex. Recent evidence shows that ACTH acts directly on lymphocytes and may even be synthesized by them (Blalock, 1989). The importance of other pituitary hormones has been seen in
hypophysectomized animals when immunocompetence is restored by treatment with prolactin (Nagy et al., 1983). In intact animals prolactin release can be blocked by treatment with bromocriptine. Contact sensitivity responses, antibody responses, the severity of EAE, T cell proliferative responses to lectins, secretion of interferon-α (IFN-α) and tumoricidal responses by macrophages are all inhibited in bromocriptine-treated animals (Bernton et al., 1988; Nagy et al., 1983). Again, the response can be restored by treatment with prolactin or with growth hormone (GH). GH is needed for the maturation of, and subsequent proper functioning of the immune system (Baroni et al., 1969). The hypopituitary dwarf mouse (deficient in GH and thyrotropin) exhibits deficient cell-mediated immunity. This is largely correctable by administration of GH alone, and totally correctable by GH plus thyroxin (Besedovsky and Sorkin, 1977). Thus, prolactin, GH, and thyrotropin exert positive effects on the immune system.

1.2.3. Neural, endocrine and immune interactions.

Neuronal control of the hypothalamic hormonal releasing factors and ultimately the release of various pituitary hormones are mediated through a complex system, which includes the central autonomic nervous system. Neuropharmacological probes such as agonists and antagonists to central nervous system adrenergic receptors have confirmed the importance of the autonomic drive upon the HPA
axis. Studies with clonidine which is a predominantly centrally acting $\alpha_2$ agonist have been carried out in patients with central autonomic failure (Shy-Drager syndrome) and an abnormality in the secretion of GH has been reported (Thomaides et al., 1993a). Similar abnormalities have been reported in depressed patients. The influence of the autonomic nervous system upon the immune system is not only restricted to the HPA-axis. Lymphoid organs are innervated by the sympathetic nervous system (SNS) and, to some extent, by the parasympathetic nervous system. Lymph nodes, thymus, spleen, gut-associated lymphoid tissue (intestinal Peyer's patches) are abundantly supplied by autonomic nerve fibres (Felten et al., 1985) and the peripheral immunosuppression induced by the intracerebroventricular injection of CRF is partially reversed by peripheral autonomic blockade (Irwin et al., 1990). Activation of the SNS or the injection of adrenaline causes leucocytosis, lymphopenia (by sequestration), and inhibition of natural-killer-cell activity (Keller-Wood et al., 1984). Sensory neurons contain a variety of neurotransmitters and neuropeptides that can influence lymphocyte function, including substance P, vasoactive intestine polypeptide (VIP), angiotensin II, calcitonin gene-related peptide (CGRP), and somatostatin (Blalock, 1989; Pavan et al., 1986; Carr, 1992) (Table 1.1.). The thymus also secretes several immune-regulating peptides (Hall et al., 1985), and the secretion of some of them is regulated directly by the autonomic nerves or indirectly by pituitary hormones (Felten et al., 1985).
Substance P and substance K, secreted by afferent sensory-nerve terminals, stimulate immunocompetent cells to secrete inflammatory cytokines (Pavan et al., 1986).

1.2.3. Neuroregulators and receptors common to immunocompetent and neuroendocrine cells.

Lymphocytes possess β-adrenoceptors (Williams et al., 1976) which respond to noradrenaline (NA) released by sympathetic nerves within the lymphoid organs and to adrenaline (Ad), release of which is controlled by splanchnic nerve input to the adrenal medulla. B cells possess more β-adrenoceptors than monocytes and monocytes more than T cells in aggregate (Khan et al., 1986). In humans, T cells of the suppressor subset (CD8*, 9.3*) possess three times as many β-adrenoceptors as cells of the cytotoxic subset (CD8*, 9.3*) and CD4+ cells (helper cells and the effectors of delayed type hypersensitivity responses) possess very few (Khan et al., 1986). The fact that suppressor cells possess more receptors than other T-cell types might suggest that whatever control the SNS exerts over T cells will be effected primarily through suppressor-cell responses to SNS-derived signals. The number of β-adrenoceptors on lymphocytes is not fixed. Their number rises, for example, on T cells driven to proliferate in vitro by mitogenic lectins (Westly and Kelley, 1987), although a decline has been reported on splenocytes following immunization with SRBC (Fuchs et al., 1988).
Glucocorticoids reverse this down-regulation to some extent (Davies and Lefkowitz, 1984). When β-adrenergic agonists bind to β-adrenoceptors, the consequence is an abrupt increase in intracellular cyclic adenosine monophosphate (cAMP). A transient rise in cycle AMP is important in the genesis of the immune response. To start the response, cells of the immune system must be triggered to move from the resting (G₀) stage of the cell cycle into G₁, and agents that elevate intracellular cAMP can provide this signal. In later stages, elevated levels of cAMP are associated with decreased cellular proliferation and increased differentiation. Sympathetic nervous system terminals may release other biogenic amines in addition to NA. Adrenaline (Ad) (from the adrenal medulla) and serotonin (from platelets) can be taken up by and subsequently released from SNS nerve terminals. Lymphocytes possess receptors for other neurotransmitters including VIP, somatostatin, and substance P (Danek et al., 1983; Payan et al., 1983; Payan and Goetzl., 1985; Payan et al., 1984). Acetylcholine is the neurotransmitter of the parasympathetic nervous system. Muscarinic, and possibly nicotinic, acetylcholine receptors have been demonstrated on lymphocytes. Cholinergic agonists promote lymphocyte proliferation in vitro (Gordon et al., 1970; Richman et al., 1981; Strom et al., 1974). Substance P is released from sensory nerve endings at sites of inflammation, for example within joints in rheumatoid arthritis or in the experimental model adjuvant-induced arthritis (Levine et al., 1984). Released substance P
**Table 1.1. Immunoregulatory Effects of Several Hormones and peptides***

<table>
<thead>
<tr>
<th>HORMONE OR PEPTIDE</th>
<th>IMMUNE FUNCTION AFFECTED</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inhibitory</strong></td>
<td></td>
</tr>
<tr>
<td>Glucocorticoids</td>
<td>Lymphocyte synthesis, inflammation</td>
</tr>
<tr>
<td>Corticotropin</td>
<td>Macrophage activation, synthesis of IgG and interferon-γ</td>
</tr>
<tr>
<td>Chorionic gonadotropin</td>
<td>Activity of T cells and natural killer cells</td>
</tr>
<tr>
<td>α-Endorphin</td>
<td>IgG synthesis, T-cell proliferation</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>T-cell proliferation, inflammatory cascade</td>
</tr>
<tr>
<td>Vasoactive intestinal peptide</td>
<td>T-cell proliferation and migration in Peyer’s patches</td>
</tr>
<tr>
<td>α-Melanocyte-stimulating hormone</td>
<td>Fever, prostaglandin synthesis, secretion of interleukin-2</td>
</tr>
<tr>
<td><strong>Stimulatory</strong></td>
<td></td>
</tr>
<tr>
<td>Oestrogens</td>
<td>Lymphocyte proliferation and secretion</td>
</tr>
<tr>
<td>Growth hormone</td>
<td>Thymic growth, lymphocyte reactivity</td>
</tr>
<tr>
<td>Prolactin</td>
<td>Thymic activity, lymphocyte proliferation</td>
</tr>
<tr>
<td>Thyrotropin</td>
<td>IgG synthesis</td>
</tr>
<tr>
<td>β-Endorphin</td>
<td>Activity of T, B, and natural killer cells</td>
</tr>
<tr>
<td>Substance P</td>
<td>Proliferation of T cells and macrophages, inflammatory cascade</td>
</tr>
<tr>
<td>Corticotropin-releasing hormone</td>
<td>Lymphocyte and monocyte proliferation and activation</td>
</tr>
</tbody>
</table>

*Data adapted from Blalock 1989.
potentiates the ongoing inflammatory response by further activating the immune system.

The capacity of the nervous system to influence immune responses by direct effects upon receptors of immunocompetent cells, is further supplemented by the influence of the endocrine system which is exerted through a remarkable range of peptides, hormones and receptors classically associated with neuronal tissue or endocrine cells (Table 1.2.). Lymphocytes contain proopiomelanocortin (POMC) mRNA, which codes for corticotropin, endorphins, and α-melanocyte-stimulating hormone and immunoreactivity corresponding to each of these peptides. They also have receptors for these peptides, glucocorticoids and CRF. The expression of the POMC gene in lymphocytes, as in pituitary corticotrophs, is increased by CRF and decreased by glucocorticoids. CRF is also secreted at sites of inflammation by monocytes and has proinflammatory actions (Crofford et al., 1992). It is likely that POMC-derived peptides secreted at the local level have a role in the regulation of inflammation. For example, the intracerebral administration of α-melanocyte-stimulating hormone inhibits fever induced by IL-1 (Lipton, 1990) and can inhibit the production of prostaglandin E (PGE₂) by IL-1 stimulated fibroblasts (Cannon et al., 1986). Whether corticotropin derived from lymphocytes is important in enhancing adrenocortical secretion is controversial (Olsen et al., 1992). However, at least one case of Cushing's syndrome
caused by ectopic secretion of corticotropin by granulomatous mass has been reported (DuPont et al., 1984). Lymphocytes reportedly contain immunoreactive thyrotropin, the secretion of which is activated by thyrotropin-releasing hormone and inhibited by thyroid hormone, as occurs in the pituitary gland (Smith, 1992). Lymphocytes also contain mRNA coding for GH and prolactin and reportedly secrete these hormones (Blalock, 1989; Kelley et al., 1992; Smith, 1992). The secretion of prolactin by lymphocytes may be physiologically important because using antiprolactin antibody to inhibit the action of prolactin inhibits mixed-lymphocyte responses in vitro.

Since, the lymphocytes appear to be under the direct influence of both neural and endocrine system, interactions of these two system at this levels, could be of similar importance to that observed in the central nervous system during the initiation or maintenance of an immune reaction.

1.3. The effects of the immune system upon the nervous system.

1.3.1. Neuroimmunology.

The central nervous system itself can be involved in immune reactions arising from within the brain or in response to peripheral immune stimuli. Activated immunocompetent cells such as monocytes, lymphocytes, and
macrophages can permeate the blood-brain barrier and take up residence in the brain, where they secrete their full repertoire of cytokines and other inflammatory mediators, such as leukotrienes and prostaglandins. Microglia are activated by toxins, cytokines, antigens, and products of cell injury arising within the brain or reaching the brain from the periphery. These cells, when activated, can secrete cytokines and inflammatory mediators (Benveniste, 1992). Endothelial and smooth-muscle cells of blood vessels in the brain can also secrete cytokines such as IL-1 and IL-6 in response to circulating antigens and toxins (Pober, 1990). All aspects of the immune and complement cascade can occur in the brain, including the regulated presentation of histocompatibility markers on glial cells.

The production of cytokines in the central nervous system can lead to profound changes in neural function, ranging from mild behavioral disturbances to anorexia, drowsiness, increased slow-wave sleep, dementia, coma, and the destruction of neurones (Dinarello et al., 1993; Denicoff et al., 1987). The neuropathogenic effects of certain viruses and bacteria that do not directly invade neurones, such as the HIV (Pulliam et al. 1991) and Borrelia burgdorferi (the spirochaete responsible for Lyme disease) (Habicht et al., 1991), may be mediated by cytokines produced by activated glia and immune cells entering the brain from the circulation. Among the newly recognized mediators of neurotoxicity induced by monocytes and glia is
<table>
<thead>
<tr>
<th>HORMONE</th>
<th>SOURCE</th>
<th>COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticotropin</td>
<td>B lymphocytes</td>
<td>Stimulated by corticotropin-releasing hormone; inhibited by cortisol</td>
</tr>
<tr>
<td>Growth hormone</td>
<td>T lymphocytes</td>
<td>Stimulated by growth hormone</td>
</tr>
<tr>
<td>Thyrotropin</td>
<td>T cells</td>
<td>Stimulated by thyrotropin-releasing hormone; inhibited by somatostatin</td>
</tr>
<tr>
<td>Prolactin</td>
<td>Mononuclear cells</td>
<td></td>
</tr>
<tr>
<td>Chorionic</td>
<td>T cells</td>
<td></td>
</tr>
<tr>
<td>gonadotropin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enkephalins</td>
<td>B lymphocytes</td>
<td></td>
</tr>
<tr>
<td>Vasactive intestinal peptide</td>
<td>Mononuclear leucocytes, mast cells</td>
<td></td>
</tr>
<tr>
<td>Somatostatin</td>
<td>Mononuclear leucocytes, mast cells, polymorphonuclear leucocytes</td>
<td></td>
</tr>
<tr>
<td>Vasopressin</td>
<td>Thymus</td>
<td></td>
</tr>
<tr>
<td>Oxytocin</td>
<td>Thymus</td>
<td></td>
</tr>
<tr>
<td>Neurophysin</td>
<td>Thymus</td>
<td></td>
</tr>
</tbody>
</table>

*Data adapted from Payan et al. 1986
nitric oxide, which acts as excitotoxin by stimulating glutamate receptors (Snyder, 1992).

A number of cytokines first discovered in immunocompetent cells are synthesized by glia. These include IL-1, 2, 4, and 6 and TNF-α (Benveniste, 1992). Interleukin-1-receptor antagonist (IL-1ra) is found in the hippocampus, hypothalamus, and other brain areas, as are IL-1 and its receptors (Licinio et al., 1991). The colocalization of IL-1ra and IL-1 forms an intrinsic paracrine regulatory system. Other neuroactive cytokines include thymosin, a peptide hormone secreted by the thymus (Hall et al., 1985), and neurokinin, a neurotrophic factor that is secreted by both macrophages and neurons (Gurney et al., 1986). In addition to glia that secrete IL-1, neurons containing IL-1 are present in several areas of the brain, including the hypothalamus and hippocampus (Breder et al., 1988; Lechan et al., 1990). The distribution of receptors for IL-1, IL-2, and IL-6 has been mapped, and mRNA coding for IL-6 and its receptors has been identified in the rat brain (Schobitz et al., 1992). The synthesis of brain cytokines is activated by local tissue injury. Locally produced cytokines stimulate the proliferation of glia and migration of macrophages that phagocytose damaged cells. One of these cytokines, IL-1, stimulates the formation of the amyloid precursor peptide, the main component of the amyloid deposits found in patients with Alzheimer's disease (Altstiel et al., 1991), thus raising the possibility that the progress of the disease may
be accelerated by inflammation limited to the brain or activation of brain IL-1 by systemic inflammation. IL-1 inhibits neuronal secretion of acetylcholine (Rada et al., 1991), an important neurotransmitter in memory processing.

In multiple sclerosis (MS), a disorder which is characterized by break-down of the blood brain barrier, perivascular cuffing, inflammation and demyelination, local production and secretion of various cytokines has been demonstrated by in situ hybridisation (Woodroofe and Cuzner, 1993). These cytokines may play an active role in the demyelination and axonal degeneration but also may affect vital CNS centres of endocrine regulation. However, the production of cytokines in the neural tissue caused by injury, toxins or inflammation may not be entirely deleterious. For example, IL-1 stimulates the production of nerve growth factor, an important neurotrophic factor (Friedman et al., 1990), and exposure of severed peripheral neurons to IL-1ra inhibits their regeneration (Guenard et al., 1991). These findings suggest that the response of IL-1 to neural damage may have healing effects by stimulating the formation of locally active neurotrophic factors. The activation of cytokine-secreting cells in the central nervous system by the injection of bacterial toxin can be mimicked by systemic or intraventricular injection of IL-1 and to some extent by injections of IL-2, IL-6, and TNF-α. Cytokines induce changes in brain function in parallel with changes in liver function. The latter include increased
Fig 1.1. Schematic representation of sympathetic nervous system-neuroendocrine-immune interactions.

Following an immune stimulus (1) a variety of lymphocytes produce a spectrum of immunological mediators including cytokines (2) which enter the circulation. These soluble mediators of immune function then gain access to the CNS (3) and stimulate either directly (4) or indirectly (via endogenous cytokine (5a) or neurotransmitter (5b) production) the release of corticotropin-releasing factor (CRF) from median eminence (6). CRF subsequently passes into the hypophysial portal vessels, enters the adenohypophysis and stimulates adrenocorticotropic (ACTH) secretion (7). Circulating ACTH initiates cortisol synthesis and release from the adrenal cortex (8) which then has a plethora of negative feedback effects on the neuroendocrine (9a) and immune (9b) systems. Centrally acting IL-1 can also activate the sympathetic nervous system (5c) which has peripheral immunosuppressive effects on spleen cells via noradrenaline (NA) release (9c).

Key to abbreviations: A1, A2: noradrenergic cell groups; Ast: astrocyte; LC: locus coeruleus; MG: microglial cell; MO: macrophage; NK: natural killer cell; OVLT: organum vasculosum of the lamina terminalis; PMN: polymorphonuclear cell; PVN: paraventricular nucleus; Tc: T cell.
synthesis of gamma globulin and C-reactive protein and decreased synthesis of albumin, transtheretin, and transferrin (Dinarello et al., 1992) and have been termed the acute-phase response. Similarly, the toxin-induced changes in cerebral function can be considered the acute-phase response of the brain.

1.4. Effects of Inflammation on Neuroendocrine Function.

Cytokines, can have important roles as regulators of endocrine function (Kennedy et al., 1991). Acting in the hypothalamus, cytokines are involved in the neuroendocrine control of anterior pituitary function and in several clinically important neuroimmunomodulatory responses to inflammation. These are the activation of the HPA axis and the inhibition of the pituitary-thyroid and pituitary-gonadal axes that occur in patients with non-endocrine disorders.

1.4.1. Activation of the pituitary and adrenal gland by inflammation.

The immune system has the capacity not only to sense the presence of foreign molecules but also to communicate this information to the brain and neuroendocrine system. This interaction, described by Blalock as the "bidirectional communication" between the immune and neuroendocrine systems (Blalock, 1989), is most evident in the increase in
secretion by the pituitary and adrenal glands that follows inflammation or infection. IL-1, IL-6, and presumably, TNF-α directly stimulate the synthesis and secretion of CRF and vasopressin (VP) at the level of the hypothalamus (Sapolsky et al., 1987) (Fig 1.1.). The consequent activation of the PHA reduces the intensity of the immune response because virtually all the components of the immune response are inhibited by cortisol. Glucocorticoids are involved in alteration of the distribution of circulating lymphocytes, inhibit lymphocyte proliferation and can switch a cell-mediated immune response to one which is antibody-mediated. They inhibit all levels of the immune response from antigen presentation to effector cell function, including class II antigen and IL-2 receptor expression on T-lymphocytes (lymphoproliferation) (Gillis et al., 1979). Moreover, the actions of cortisol or other glucocorticoids include suppression of the release of interferon-γ (INFγ); granulocyte-monocyte colony-stimulating factor (GMCSF); IL-1, 2, 3, and 6; TNF-α; inflammatory mediators such as eicosanoids, bradykinin, serotonin, and histamine; and tissue-destroying enzymes, including collagenase and elastase (Munck et al., 1984). Reduction of eicosanoid production in certain immune cells, such as macrophages, is realised through the induction and redistribution of the phospholipase A₂ inhibitory protein, lipocortin 1 (Elderfield et al., 1990). Distributed throughout nervous and endocrine tissue (Smith et al., 1993), lipocortin 1 is also implicated in the negative feedback effects of glucocorticoids on the
hypothalamus and pituitary glands (Smith et al., 1993). Increased levels of lipocortin 1 in the spinal cord and cerebellum of Lewis rats with EAE coincide with simultaneous rise in plasma corticosterone concentration (Bolton et al., 1990). Lipocortin 1 is also raised in white matter and plaque tissue from patients with multiple sclerosis (Elderfield et al., 1990).

Support for the hypothesis that an important function of the pituitary-adrenal response is to prevent over-exuberant immune reactions has come from the study of immune responses in a strain of rats with a genetic defect in the synthesis of CRF. These rats, of the Lewis strain, do not respond to stress or inflammation with an increase in hypothalamic CRF mRNA or in adrenocortical secretion. Acute arthritis develops when these rats are injected with suspensions of streptococcal cell walls, whereas it does not develop in rats of Fischer strain, in which the responses of the hypothalamus, pituitary gland, and adrenal glands are normal. (Sternberg et al., 1989; Sternberg et al., 1992). However, similar responses are induced in Fischer rats where pituitary and adrenal function is suppressed. Administration of glucocorticoids to Lewis rats suppresses the inflammation.

The relevance of the pituitary-adrenal response to inflammation in humans is being evaluated. As compared with patients who have osteoarthritis, patients with rheumatoid
arthritis have a less pronounced diurnal rhythm of cortisol secretion and less pronounced pituitary and adrenal responses to surgery, despite having similar increases in the circulating concentrations of IL-1β and IL-6 (Chikanza et al., 1992). Reduced pituitary and adrenal responses to CRF and slightly reduced plasma cortisol concentrations have been reported in patients with chronic fatigue syndrome (Demitrack et al., 1991). Patients with depression have reduced pituitary and adrenal responses to CRF, although their plasma cortisol concentrations are characteristically slightly elevated.

1.5. Neural and endocrine responses in EAE.

Changes in neural and endocrine parameters, known to exert feedback control on immunological reactivity, have been observed during the pre-clinical and clinical stages of primary acute or adoptively transferred EAE (Leonard et al., 1991, Mackenzie et al., 1989). Splenic NA levels fall significantly during the period preceding and coincident with the preclinical lymphoproliferative stage of the immune response and a rise in serum corticosterone is observed. An associated increase in splenic lymphocyte β-adrenoceptor density may occur in response either to lymphoproliferation or negative feedback, or be heterologously related to the increase in circulating corticosterone. The stress of clinical signs of EAE then leads to highly significant increases in splenic NA and plasma glucocorticoids, both
immunosuppressive agents. Circulating glucocorticoid levels are closely correlated with the clinical score in EAE and represent an important recovery mechanism. Central control of the HPA loop is demonstrated by the tight inverse correlation between serum corticosterone and hypothalamic NA (Leonard et al., 1991). Disruption of the HPA loop and of sympathetic innervation both peripherally and centrally has wide-ranging effects on the response of the immune system to the antigenic challenge of EAE (Leonard et al., 1991). In all experimental groups with intact peripheral and/or central noradrenergic pathways the uniformly increased splenic NA content observed at height of disease activity appears to contribute to recovery. This is in agreement with the observation that isoproterenol, a β-adrenergic agonist ameliorates the clinical signs in EAE (Chelmicka et al., 1989). Chemical sympathectomy with 6-hydroxydopamine intraperitoneally (i.p.) results in depletion of NA in lymphoid organs as the drug does not cross the intact BBB. The same treatment i.c.v. leads to depletion in the CNS only. The clinical signs of EAE are exacerbated and recovery rate is delayed when peripheral noradrenergic innervation is interrupted, reflecting the 90% depletion of lymphoid tissue NA despite the fact that circulating CS was elevated. In contrast clinical disease is ameliorated when central NA is depleted, the apparent result of serum corticosterone levels raised preclinically which persist throughout the time period under investigation. Adrenoceptor supersensitivity or abrogation of glucocorticoid feedback may account for these
findings. The overriding immunoregulatory influence of glucocorticoids is emphasised by the rapid onset of clinical EAE and morbidity in adrenalectomised animals (Mason et al., 1990). However, the strong inverse correlation found between hypothalamic NA and circulating CS indicates that regulation of the HPA-axis may ultimately be controlled by central sympathetic pathways.

Neural and hormonal modulation of the cell mediated immune response during preclinical and clinical EAE appear therefore to operate on two levels. The first represents the response to the general immune challenge and may have a regulating influence on the extent and duration of lymphocyte proliferation both in EAE and adjuvant animals. This stage is followed by the onset of symptoms of disease, the trauma and stress of which introduce hormonal and neurotransmitter signals, possibly constituting a recovery mechanism. These regulatory factors which could be influenced by both intrinsic and extrinsic events may have an important role in controlling the onset of relapses and remissions in chronic EAE and, by inference, in multiple sclerosis.

1.6. Neural, endocrine and immune interactions in MS.

Multiple sclerosis plaques can potentially disrupt CNS pathways controlling endocrine or sympathetic nervous system output and thereby affect immune responses. In addition, the
activated lymphocytes seen in MS (Reder et al., 1985) could produce cytokines and ACTH which would alter or amplify the immune abnormalities that are present in MS.

1.6.1. The hypothalamic-pituitary-adrenal (HPA) axis and ACTH in MS.

In MS, serum ACTH levels may be higher than normal (Allen et al., 1980; Nowak et al., 1987). Baseline serum cortisol levels are normal overall (Maida et al., 1979; Reder et al., 1987; Teasdale et al., 1967), but some series contain a disproportionate number of MS patients with high serum cortisol (Kaschka et al., 1980; Millac et al., 1969; Trotter et al., 1980). Cortisol response to intravenous ACTH seems to be blunted (Maida et al., 1979) and quite variable (Sullivan et al., 1987) compared to normal controls, possibly due to down-regulation of adrenal responses by chronically elevated ACTH. Glucocorticoid metabolism in some patients with MS may be more rapid than normal (Reder et al., 1987), which may explain in part the variable responses to ACTH therapy observed in MS patients.

Feedback regulation of the HPA axis can be studied with the dexamethasone suppression test (DST). A total of 50% of patients with active MS are non-suppressors compared to 5% of controls (Reder et al., 1987). This subgroup does not show a significant reduction in cortisol secretion after dexamethasone, and the high frequency of non-suppression is
equivalent to that seen in major (endogenous) depression. The MS non-suppressor group, compared to suppressors, is not more depressed. The attenuated HPA response to glucocorticoids is only partially explained by the increased rate of dexamethasone breakdown in MS non-suppressors and may perhaps reflect lowered sensitivity to feedback regulation by the HPA axis. In summary, plasma ACTH may be elevated, cortisol levels are normal or increased, and glucocorticoids are metabolized more rapidly in some patients with MS compared to controls. In functional tests, there are blunted responses to ACTH and to dexamethasone. This could be the result of chronic high levels of ACTH or glucocorticoid which cause desensitization (down-regulation) of their responses. Alternatively, MS plaques near the third ventricle could disrupt feedback regulation of ACTH secretion if stimulatory CRF pathways were damaged. Finally raised ACTH could be derived from non-pituitary sources independent of CNS control. "Ectopic" ACTH is produced by tumours such as small-cell carcinomas of the lung and bronchial carcinoid, but this ACTH is often not bioactive. Cushing's syndrome, however, has resulted from a high molecular weight precursor form of ACTH secreted by an acute myeloblastic leukaemia (Pfluger et al., 1981). "ACTH-like" substance from activated lymphocytes is bioactive and can stimulate the adrenal gland (Smith et al., 1982). It is likely that non-ACTH segments of the POMC molecule are also secreted by lymphocytes and could affect adrenal responses to ACTH. However, whether or not these products are produced
in excess from mononuclear cells in MS patients in relapse or remission of the disease remains to be determined.

1.6.2. Sex Hormones and MS.

MS is more frequent in women than in men. Only in one of 30 surveys from 18 countries was there a male predominance (Duquette et al., 1992). The overall sex ratio (expressed as the number of women over the number of men) was 1.8 to one. In both the early and late onset groups, the sex ratio increased to three to one; this increase was related at least temporally to puberty and the menopause. Both prolactin and oestrogens may have immunomodulatory effects which influence the course of MS. Prolactin binds to specific receptors on mononuclear cells and enhances immune responses such as mixed lymphocyte and graft versus host reactions (Bernton et al., 1988; Hiestand et al., 1986), macrophage tumoricidal activity, T-cell proliferation, and IFN-γ secretion (Bernton et al., 1988). Prolactin levels average 13ng/ml serum in men and women, but are 50-200 ng/ml during the last two trimesters of pregnancy and rise further during the act of nursing and also rise during stress (Martin et al., 1987). Flares of MS are less common during pregnancy (Birk et al., 1986; Nelson et al., 1988), and this is followed by increased risk for exacerbation postpartum, with no subsequent net effect of pregnancy on the disease (Tillman, 1950). However, prolactin levels do not seem to play a role in the relative increase in the frequency of MS
attacks during the six postpartum months, since there is no significant difference in the exacerbation rate in mothers with MS who breastfeed (38%) compared to those who do not (31%) (Nelson et al., 1988). Oestrogens enhance humoral immune responses and some autoimmune diseases, but inhibit most T-cell mediated responses (Grossman, 1984). During pregnancy, oestrogens and progesterones increase 100-fold. Oestrogen receptors are not found on CD4 cells (helper/inducer) but are present on CD8 cells (suppressor/cytolytic) (Cohen et al., 1983). The CD8 subset is implicated in many of the immune abnormalities seen in MS (Reder et al., 1985). Oestradiol enhances B-cell differentiation and Ig secretion in vitro by inhibiting suppressor cells (Paavonen et al., 1981). Oestradiol also inhibits EAE, whereas methoxyprogesterone augments the severity of EAE (Arnason et al., 1969). It should be noted however that oral contraceptives have not been shown to affect the course of MS (Poser et al., 1979).

Finally, MS plaques around the ventricles and in the hypothalamus could disrupt the integrity of circuit pathways regulating the gonadotrophic factors and hormones.

1.6.3. The sympathetic nervous system (SNS) in MS.

The SNS innervates the spleen, lymph nodes, the adrenal and thymus (Felten et al., 1987). Noradrenaline (NA) is the principal catecholamine released by the SNS. It can affect immune functions either through the proximate contact
of nerve terminals with mononuclear cells in immune organs or after its release into the circulation. 6-OHDA destruction of the SNS causes an acute rise in serum catecholamine levels followed by a marked fall. If the SNS (and some CNS neurons) of mice are permanently destroyed at birth with 6-OHDA (sympathectomy), the levels of NA in the spleen fall. Eight weeks after neonatal sympathectomy, levels of NA are 1.25ng/g compared to 485±70 ng/g of spleen tissue in the control animals (Miles et al., 1985). Destruction of the SNS in adulthood also causes a drop in splenic NA levels to 58±9ng/g several weeks after 6-OHDA treatment. If 8 weeks are allowed to elapse after sympathectomy (to avoid the effects of the surge in catecholamines immediately after SNS destruction), spleen cells are more responsive to mitogens (Miles et al., 1985) and antibody responses are sometimes increased compared to controls (Besedovsky et al., 1983; Miles et al., 1981; Williams et al., 1981). As mentioned above, sympathectomy increases the severity of EAE and experimental allergic myasthenia gravis (Agius et al., 1987).

Several phenomena seen in MS parallel these animal models of SNS destruction. In MS, plaques in the CNS sympathetic pathways are presumably the cause of SNS damage. SNS regulation of heart rate in MS is less effective than in controls (Neubauer et al., 1978). The sympathetic skin response to electrical stimulation is present in all controls but is absent in 50% of chronic progressive MS
patients (Karaszewski et al., 1990). In addition, \( \beta \)-adrenoceptors on lymphocytes are increased in patients with progressive MS (Karaszewski et al., 1990). The most pronounced \( \beta \)-adrenoceptor increase is seen in the CD8 subset- the ineffectual "suppressor" subset that is largely responsible for the low ConA suppressor function and high PWM-induced Ig secretion observed in MS (Reder et al., 1985). NA decreases most immune responses in vitro (Kammer, 1988) (although under certain conditions enhanced immune function can occur). An increase in \( \beta \)-adrenoceptors on CD8 cells might cause these suppressor cells to be very sensitive to fluctuations in serum NA. In summary, there is compelling evidence that SNS function is abnormal in MS. Some of the consequences of SNS destruction in animals, such as elevated lymphocyte \( \beta \)-adrenoceptors, are also present in the chronic progressive form of the disease and could affect immunity.

### 1.7. Aims of this thesis.

The recent developments in monitoring disease activity with Magnetic Resonance Imaging (MRI), have shed light on the clinical forms of MS. It appears that benign and primary progressive MS patients have a lower CNS lesion load (Thompson et al. 1991). Are there recovery mechanisms involving neural-endocrine function which can contribute to the diversity of the disease presentation? Studies in MS patients, so far, have failed to produce substantial
evidence of a defective neural-endocrine and immune function responsible for this variable presentation of the disease. However, a direct investigation of the relationship between disease activity (recorded by MRI), and neural-endocrine and immune function could clarify the importance of these systems in recovery.

Although there is evidence of autonomic dysfunction in the late stages of progressive MS, to what extent this may contribute to the immunopathogenesis of the disease remains unclear. Certainly, no immune abnormality has been described in patients with peripheral or central autonomic dysfunction. However, it is unknown whether a defective autonomic nervous system may influence the outcome of an autoimmune disorder by affecting recovery mechanisms operating through the neural-endocrine-immune network.

Though it is uncertain whether MS is systemic disorder or is confined to the CNS, correlations between disease activity and alterations in the activity and phenotype of PBMCs may further clarify this issue. Since immunocompetent cells, by virtue of their receptors for neurotransmitters and various hormones, are under the direct influence of the neural and endocrine function, a study of the interactions of these systems at this level may reveal important immunoregulatory mechanisms or provide valuable markers of the disease activity. Aspects of these interactions have been studied and are presented in this thesis. In
particular, the aims of this thesis have focused on:

(1) The investigation of $\beta$-adrenoceptor expression on peripheral blood mononuclear cells in multiple sclerosis and other autoimmune or putative autoimmune disorders such as myasthenia gravis and rheumatoid arthritis. The elucidation of the effect of immune mediators upon the expression of lymphocytic $\beta$-adrenoceptors.

(2) The in vitro regulation of IL-2 receptor (IL-2R) expression through the $\beta$-adrenoceptor second messenger system cAMP, in lymphocytes from normal subjects and MS patients.

(3) The possible correlation of alterations in lymphocytic $\beta$-adrenoceptor expression with disease activity of MS assessed by clinical criteria and Magnetic Resonance Imaging, in a longitudinal study. The exploration of a possible link between increased $\beta$-adrenoceptor expression and the activated state of lymphocytes (in particular expression IL2-R) in MS.

(4) The study, by physiological and neuropharmacological means, of the peripheral and central autonomic nervous system and its interaction with the endocrine system in MS. The delineation of the possible contribution of autonomic dysfunction in MS to the increased lymphocytic $\beta$-adrenoceptors.
2.1.1. β-adrenoceptor subtypes.

The β-adrenoceptor subtypes are coupled by G protein to adenylate cyclase and produce alterations in cellular activity by raising intracellular levels of cAMP. The \( \beta_1 \)- and \( \beta_2 \)-adrenoceptors have been well characterized pharmacologically (Arnold et al., 1966; Lands et al., 1967), and more recently the use of molecular biology techniques has provided new information about the structure and functional domains of the receptor (Dixon et al., 1988; Tota et al., 1991). Pharmacologically, the classical \( \beta \)-adrenoceptors have been defined as receptors at which (−)-isoprenaline acts as an agonist and (−)-propranolol as an antagonist. Recent studies in the rat soleus muscle indicate that (−)-\(^{125}\text{I}\)-cyanopindolol in the presence of propranolol can identify atypical \( \beta \)-adrenoceptors in homogenate binding and autoradiographic experiments (Molenaar et al., 1992). Using the molecular approach cloning and sequencing \( \beta_3 \)-adrenoceptors from several species has been successful.
The β-adrenoceptor subtypes are among the best defined of the G protein-linked superfamily of receptors. There are at least three human genes that express separate β-adrenoceptor subtypes, the β₁-, β₂-, and β₃-adrenoceptors (Chung et al., 1987; Dixon et al. 1987a,b; Frielle et al., 1987; Kobilka et al., 1987a,b; Emorine et al., 1989). The β₁- and β₂-adrenoceptors show 48.9% homology, and the β₃-adrenoceptor shows 50.7 and 45.5% homology with the other two receptors, respectively (Emorine et al., 1989). The β₁ and β₂ receptors are classified according to the order of agonist potency in competing with antagonist binding, the order being adrenaline = isoprenaline > noradrenaline, and isoprenaline> adrenaline> noradrenaline for β₁ and β₂ receptors, respectively.

A great deal of work has been done with the β₂-adrenoceptor to determine the functional domains (Dixon et al., 1988; Dohlman et al., 1991; Strosberg, 1991) (Fig 2.1.). The hydrophobic regions of the receptor contained in the transmembrane-spanning regions are essential for ligand binding and protein folding (Tota et al., 1991). Studies with chimeric receptors have shown that the VI and VII transmembrane-spanning regions (Fig 2.1.) are important in determining the specificity of antagonist binding (Frielle et al., 1988; Kobilka et al., 1988). However, interaction of β-adrenoceptors with G stimulating protein involves residues of the third intracellular hydrophillic loop of the receptor (Strader et al., 1987;
Fig 2.1. Functional domains of the $\beta_2$-adrenoceptor.

Large-diameter segments represent the transmembrane-spanning regions (I-VII); the upper portion of the structure represents the extracellular and the lower portion the intracellular domains. $\beta$-ARK, $\beta$-adrenoceptor kinase (From the Journal of Neurochemistry 1993; Vol. 60, No 1. p 15).
O'Dowd et al., 1988; Cheung et al., 1989).

Phosphorylation of the β-adrenoceptor is closely involved in the desensitization phenomenon. Several protein kinases are known to be involved, including protein kinase A (PKA), protein kinase C (PKC), and β-adrenoceptor kinase. PKA and PKC phosphorylate the receptor, which may be involved in heterologous desensitization. β-Adrenoceptor kinase only phosphorylates agonist-occupied receptors and is believed to be responsible for homologous desensitization.

2.1.2. Localization of β-adrenoceptors.

β-Adrenoceptors were first identified in the mid-1970s by radioligand binding studies in frog erythrocyte membranes (Lefkowitz et al., 1974) and in the cardiac tissue (Harden et al., 1975). They were subsequently demonstrated in various cell lines (Maguire et al., 1976), found to be present in many animal tissues such as adipocytes (Williams et al., 1976), brain (Bylund et al., 1976) and lung (Blanck et al., 1979), and characterized in human granulocytes (Galant et al., 1978) and lymphocytes (Williams et al., 1976). The receptors in leucocytes were then studied uniquely as an easily accessible model, mirroring cardiovascular and lung adrenergic receptors (Motuisky et al., 1982).

Since 1974, antagonist binding with $^3$H-alprenolol (Lefkowitz et al., 1974) or $^{125}$I-labelled cyanopindolol (ICYP) (Brodde et al., 1981) has been used to detect β-adrenergic receptors in all tissues. The hydrophillic ligand $^3$H-CGP 12177 which binds only to those receptors on the cell surface (Hertel et al., 1983), has helped to determine receptor redistribution after agonist treatment in vitro or in vivo (Motulsky et al., 1986; DeBlasi et al., 1986; Landmann et al., 1988). Recently, photoaffinity labelling of β-adrenoceptors has been developed (Ruoho et al., 1987), and monoclonal anti-idiotypic antibodies directed against the antagonist alprenolol have been shown to bind to the receptor (Strosberg et al., 1989). Studies with antagonists conjugated to fluorescein did not permit satisfactorily sensitive measurements because of low affinity and high non-specific binding.

Radioligand studies in human mononuclear leucocytes revealed the predominant presence of $\beta_2$-adrenoceptors subtype (Landamann et al., 1984). In resting cells, two classes of receptors were shown to exist with a low and a high affinity for the agonist (Kent et al., 1980). Short-lasting agonist stimulation in vitro gave rise to a single population of receptors with low agonist affinity,
indicating that the receptor had been phosphorylated after uncoupling from the regulatory G-protein and catalytic unit (Lefkowitz et al., 1988; De Blasi, 1989; Benovic et al., 1986). Prolonged agonist stimulation in vitro resulted in a decrease in receptor number due to internalization and potential receptor degradation (Sibley et al., 1985; Motulsky et al., 1986).

2.1.4. Distribution of β-adrenoceptors in lymphocyte subsets and monocytes.

The results obtained by several different research groups are consistent with respect to the differential distribution of β-adrenoceptors in lymphocyte subsets. B cells express more receptors than T cells (Landmann et al., 1984; Pochet et al., 1979; Krawietz et al., 1982; Paietta et al., 1983; Griese et al., 1988; Van Tits et al., 1990). Among the T cells, those with CD8 phenotype have more receptors than those with the CD4 phenotype (Landmann et al., 1988; Landmann et al., 1984; Van Tits et al., 1990; Khan et al., 1986; Maisel et al., 1990). According to Khan et al., receptor density is higher on CD8 cells with a suppressor phenotype than on those with a cytotoxic phenotype. Natural killer (NK) cells defined by antibodies against CD57 (Leu 7) or CD56 (Leu 19) present more receptors than CD8 or CD4 cells in the two studies where these cells were compared (Van Tits et al., 1990; Maisel et al., 1990). According to Maisel et al.,
the affinity for iodopindolol did not significantly differ between the different subsets. In monocytes, two groups using different isolation procedures found similar receptor numbers (Van Tits et al., 1990; Maisel et al., 1990).

2.1.5. Methods

2.1.6. /β/-adrenoceptor binding on peripheral blood mononuclear cells using (-)-125I cyanopindolol (ICYP).

Binding assays on human lymphocytes have been performed with the β-adrenergic radio-ligands (-)-3H-dihydroalprenolol (3H-DHA) and (±)-125I hydroxybenzylpindolol (125IHYP). However, neither 3H-DHA nor 125IHYP seem to be ideal ligands for this purpose. 3H-DHA is a pure (-)-isomer with an affinity in the nanomolar range and a specific activity of 20-60 Ci/m mole. This relatively low specific activity necessitates the use of large amounts of blood, which is a disadvantage in long-term studies.

IHYP is a racemate with an affinity for β-adrenoceptors in the 10^{-10}-10^{-11} molar range and a radioactivity of 2175 Ci/m mole. Although the affinity of this ligand to β-adrenoceptors is about 100 times greater than that of 3H-DHA, IHYP also has some disadvantages. A high non-specific binding of IHYP has been described in
several tissues, which can be depressed only partly by high concentrations of phentolamine or serotonin. This effect on IHYP binding is most likely nonspecific binding. The highly specific β-adrenergic radio-ligand, \((-)\)-\(^{125}\)I cyanopindolol (ICYP) has been introduced in β-adrenoreceptor binding assays which appear to have high affinity and specificity to β-adrenoceptors in peripheral and central tissues. In contrast to IHYP, ICYP-binding was found to be unaffected by phentolamine or serotonin and it exerts a low non-specific binding, even in the absence of phentolamine (Engel et al., 1981). Therefore, for this study ICYP was considered the ideal ligand for identification and characterization of β-adrenoceptors on PBMCs.

2.1.7. Isolation of Peripheral Blood Mononuclear Cells (PBMCs).

Peripheral blood was drawn from healthy volunteers (aged 23-70 years) by venipuncture into heparinized 10 ml Vacutainer tubes. PBMCs were isolated according to the method of Boyum (1968). The mononuclear layer was washed twice in phosphate-buffered saline solution (PBS). Viability was 95% as assessed by eosin (2% wt/vol) exclusion.
2.1.8. Binding characteristics of (−)-125I cyanopindolol (ICYP) to intact PBMCs.

PBMCs were washed in PBS and resuspended at a concentration of 5\times10^6 cells/ml. The ligand-binding assay was carried out in triplicate in round-bottom 96-well plates using the β-adrenergic antagonist [125I]CYP (Amersham, Aylesbury, UK), specific activity 2000 Ci/mmol. Total binding was determined by incubating 5\times10^5 cells in a 200μl volume with seven concentrations (range 10–300 pM) of [125I]CYP for 90 minutes at 30°C. "Non-specific" binding was determined over the same concentration range of [125I]CYP in the presence of 1μM (−)-DL-propranolol (Sigma, Poole, UK). "Specific" binding of ICYP is defined as total radioactivity minus non-specific binding. After incubation the cells were harvested using a Titertek cell harvester on filter mats (Skarton, Newmarket, UK). The mats were dried and the discs counted for 60 seconds in a LKB mini-gamma counter at an efficiency of 80% (Pharmacia-LKB, Milton Keynes, UK). Binding kinetics were determined by Scatchard analysis (Scatchard, 1949) using an EBDA/Ligand computer program (Munson and Rodbard, 1980). The regression lines were calculated by the least squares method. The equilibrium dissociation constant (K_D) is expressed in pM and the maximal number of binding sites (B_max) is expressed in sites/cell after conversion with Avogadro's number:
sites/cell = \( B_{\text{max}} \times (6.023 \times 10^{23}) \)/ no. of cells per litre

"Specific" binding of ICYP increased linearly with increasing concentration of cells from \( 1 \times 10^5 \) to \( 8 \times 10^6 \) cells/assay. Binding however usually performed at a concentration of \( 5 \times 10^5 \) cells/assay. The "specific" binding of ICYP rose with increasing ICYP concentrations amounting to 60% (at 10-80 pM), 30% (at 150 pM) and 15% (at 300 pM) of total ICYP binding (Fig 2.2.).

2.1.9. Kinetics for \(^{125}\text{I}-\text{CYP} \) binding.

The time course for binding was determined by incubating \( 5 \times 10^5 \) cells with 160 pM \(^{125}\text{I}-\text{CYP} \) in the absence and presence of 1\( \mu \text{M} \) (-)-DL-propranolol at 30\(^\circ\)C. Specific binding was determined in triplicate at various times after the addition of the \(^{125}\text{I}-\text{CYP} \) and expressed as cpm (Fig 2.3.).
Fig. 2.2. Binding Characteristics of $\beta$-adrenoceptors.

(A) Binding characteristics of $^{125}$I-CYP to PBMCs ($5 \times 10^6$ cells/ml). The assay was carried out at 30°C for 90 mins in 96 well plates. Non-specific binding was determined in the presence of 1 $\mu$M (-)-propranolol. (B) Scatchard analysis revealing a linear plot indicating a single receptor population with a $K_D$ of 90pM and approximately 930 sites per cell. (Each point represents the mean value of three measurements. The intra-assay variation was less than 5%).
PBMCs (4×10^6 cells/ml) were incubated at 30°C in round bottom 96 well plates with 160 pM ^125^I-CYP with and without 1μM (-)-propranolol. At times indicated, the cells were harvested and the mean total and non-specific binding from triplicate wells expressed as counts per minute (CPM). Equilibrium of non-specific displacement was observed after 45 mins. (Each point represents the mean value of three measurements. The intra-assay variation was less than 5%).
2.1.10. Competition for $^{125}$I-CYP binding with (−) propranolol, atenolol, ICI 118551 and phentolamine.

PBMCs (5 x $10^5$) per assay were incubated for 90 min at 30°C with 160 pM $^{125}$I-CYP in the presence of increasing concentrations of the unlabelled competitors ([−]-dl-propranolol (β-antagonist), atenolol (β₁-antagonist), ICI 118551 (β₂-antagonist) and phentolamine (α-antagonist). Each concentration was carried out in triplicate and the amount of $^{125}$I-CYP bound expressed as counts per minute (CPM) (Fig 2.4.).

2.1.11. Binding characteristics of (−) $^{125}$I-CYP in concentrations above 300 pM.

The binding characteristics of (−) $^{125}$I-CYP over a range of concentrations from 10 to 500 pM were tested. Saturability of binding was observed up to 300 pM concentrations. However, above 300 pM the binding was non-saturable and non-specific indicating detection of non β-adrenoceptor sites. CYP is a lipophilic ligand which crosses the membrane and binds to intracellular sites and in high concentrations (above 300 pM) it appears to be non-specific in binding. This was further supported by the abolition of this binding in the presence of phentolamine ($10^{-4}$M) (Fig 2.5.).
2.1.12. Results.

β-adrenoceptor sites were determined in 50 healthy subjects aged 20-70 years (10 subjects aged 20-30 years; 30 subjects aged 30-60 years; 10 subjects aged 60-70 years). β-adrenoceptor numbers and affinity ($K_p$) expressed in mean ± sem are presented in Table 2.1.1. There is a slight decline of the number of receptors per cell with age.

**Table 2.1. β-adrenoceptor densities and affinity($K_p$)(mean ± s.e.m.) on PBMCs in normal subjects.**

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Normal (N=50)</th>
<th>Subjects (N=10)</th>
<th>Subjects (N=30)</th>
<th>Subjects (N=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-adrenoceptors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(sites/cell)</td>
<td>1192±77</td>
<td>1400±140</td>
<td>1240±41</td>
<td>950±50</td>
</tr>
<tr>
<td>$K_p$(nM)</td>
<td>107±14</td>
<td>120±13</td>
<td>110±18</td>
<td>94±10</td>
</tr>
</tbody>
</table>
Fig. 2.4. Competitive Binding of β-adrenoceptors.

PBMCs (4x10^6 cells/ml) were incubated in triplicate for 90 min at 30°C with 160pM ¹²⁵I-CYP in the presence of increasing concentrations of unlabelled competitors. The amount of ¹²⁵I-CYP bound was calculated as a percentage of the total counts per minute. (Each point represents the mean value of three measurements. The intra-assay variation was less than 5%).
Fig. 2.5. $^{125}$I-CYP binding between 10 to 500 pM.

Percentage of specifically bound $^{125}$I-CYP over a range of concentrations from 10 to 500 pM. Note that binding above 300 pM $^{125}$I-CYP becomes nonsaturable. (Each point represents the mean value of three measurements. The intra-assay variation was less than 5%)
2.1.13. Discussion

The method for the β-adrenoceptor assay was adapted from a previous assay system used to determine β-adrenoceptors on human lymphocytes (Brodde et al., 1981). Due to the small volume of the assay (200μl total) it was possible to carry out the whole assay in round bottom 96 well plates. This reduced both the preparation and harvesting times allowing up to eight assays to be run at once. $^{125}$I-CYP over a concentration range of 10 to 300 pM demonstrated stereospecific saturable binding with a high affinity for β-adrenoceptors and virtually no affinity for α-adrenoceptors. This was evident in the competition studies where displacement profiles for propranolol and ICI 188551 were similar while phentolamine was only capable of displacing $^{125}$I-CYP binding at concentrations above $10^{-4}$M. The binding curves for viable intact PBMCs revealed a saturable single site receptor population with a $K_b$ of 120 pM and approximately 1200 sites per cell. A slight reduction in the number was observed with aging which is in keeping with previous observations (Schocken et al., 1977).

However, at higher concentrations of $^{125}$I-CYP (>300 pM) a second class of binding site was labelled. The appearance of these low-affinity binding sites seems to be due to an uptake of the lipophilic ligand into the cell.
Such an uptake is not restricted to lymphocyte β-adrenoceptors, but seems to be a common problem in intact cell studies. For example, Maloteaux (1983) have shown that $^3$H-spiroperidol or $^3$H-haloperidol labelling of intact lymphocytes, fibroblasts, hepatocytes and neuroblastoma cells was not displaced by dopamine receptor antagonists, but were very sensitive to chloroquine, known to be a lysosomotropic drug. These results suggest that chloroquine prevents the accumulation of the lipophilic ligands in the lysosomal compartment. In addition to chloroquine, phentolamine has been useful in intact cell studies as an inhibitor of this ligand-uptake phenomenon (Meurs et al., 1982). In the present study addition of phentolamine ($10^{-4}$M, a concentration which did not significantly affect ICYP binding) to the incubation medium abolished the low affinity component. These results favour the idea that in fact the low affinity binding site is due to uptake of the ligand into the cell.

In conclusion, it appears that concentration range of 10 to 300 pM $^{125}$I-CYP is appropriate for detection of β-adrenoceptor sites of high affinity.
2.2. β-adrenoceptor second messenger system.

2.2.1. G Proteins.

All known adrenoceptors are coupled to their effector systems by guanine nucleotide binding proteins (G proteins) (Birnbaumer, 1990). All families of those proteins contain three subunits, the β and γ subunits of 35-37 and 8-10 kDa and α subunits of 45-52 kDa.

Several distinct G proteins are involved with adrenoceptor effector coupling. The α subunits possess GDP and GTP binding sites with affinities that vary with the occupation of adrenoceptors. They also have GTPase activity, and most have ADP ribosylation sites for cholera or pertussis toxin (Gilman, 1987). Various forms of G proteins capable of stimulating (Gα) or inhibiting (Gγ) the adenylate cyclase have been isolated.

2.2.2 Adenylate Cyclase.

The intracellular levels of the second messenger cAMP are influenced by the activity of adenylate cyclase, which is in turn controlled by the activation of membrane-bound receptors and the signal-transducting G proteins. Biochemical (Pfeuffer et al., 1985; Mollner and Pfeuffer, 1988) and molecular biological (Krupinski et al., 1989)
evidence demonstrates the existence of several forms of adenylate cyclase with molecular masses in the range 115-150 kDa. The most common form is a glycoprotein with a molecular mass of 115 kDa, which can be partly purified from brain using a forskolin-affinity column, and further purified using wheat germ agglutinin or calmodulin affinity columns. These preparations, when reconstituted with β-adrenoceptors and Gs in phospholipid vesicles, are capable of hormone-dependent synthesis of cAMP (May et al., 1985; Feder et al., 1986). The main tool used for the localization of adenylate cyclase is the diterpene forskolin. Forskolin stimulates adenylate cyclase directly and, at much lower concentrations, enhances cAMP accumulation in response to stimulation of Gs-linked receptors. [3H]-Forskolin binds with high affinity to adenylate cyclase, and there is an excellent correlation between the ability of analogues to stimulate adenylate cyclase and inhibit [3H]forskolin binding (Laurenza et al., 1989).

2.2.3. cAMP-dependent protein kinase.

cAMP-dependent protein kinase is a major enzyme mediating the phosphorylation of target proteins leading to metabolic and physiological responses in cells. This enzyme is a tetramer with two regulatory (R) and two catalytic (C) subunits. The R subunits bind to cAMP and cause the tetramer to dissociate, which then catalyzes
ATP-dependent phosphorylation of proteins to alter cell activity. There are at least two isoenzymes which differ in their localization and transmitter specificity (Gundlach and Urosevic, 1989). Membrane-bound cAMP-dependent protein kinase has been localized using [\textsuperscript{3}H]cAMP.

2.2.4. Adenosine 3':5'-Cyclic Monophosphate Assay.

In the 30 years since the discovery of cAMP (Sutherland et al., 1958) two types of assay to measure the cAMP content of the cells have been described, i.e enzymatic assays (Butcher et al., 1965) and competitive binding assays based on either binding proteins (Gilman, 1970) or specific antibodies (Steiner et al., 1969). The sensitivity and performance of the competitive binding assays have improved considerably in the course of time (Harper et al., 1975; Amersham Life Science, 1990).

In this study, production of intracellular lymphocytic cAMP was measured using a commercial kit (Amersham, U.K.). The assay is based on the competition between unlabelled cAMP and a fixed quantity of \textsuperscript{125}I-labelled cAMP for a limited number of binding sites on a cAMP-specific antibody. With fixed amounts of antibody and radioactive ligand, the amount of radioactive ligand bound by the antibody will be inversely proportional to the concentration of added non-radioactive ligand:
The antibody bound cAMP is then reacted with the Amerlex-M second antibody reagent which contains second antibody bound to magnetizable polymer particles. Separation of the antibody bound fraction is effected by either magnetic separation or centrifugation of the Amerlex-M suspension and decantation of the supernatant. Measurement of the radioactivity in the pellet enables the amount of labelled cAMP in the bound fraction to be calculated. The concentration of unlabelled cAMP in the samples is then determined by interpolation from a standard curve.

2.2.5. Measurements of intracellular cyclic AMP.

PBMCs from five normal subjects were washed twice in a 50mM Tris-hydrochloric acid buffer, pH 7.4, containing 0.9% sodium chloride, 4 mM ethylenediaminetetraacetic acid (EDTA), and 8 mM theophylline. Both theophylline and EDTA strongly inhibit phosphodiesterase activity, and are used in the assay in order to prevent the breakdown of cAMP. Cells in different concentrations (5 to 80 x 10⁵ per incubation) were suspended in 0.2 ml of the same buffer. The cell suspensions were incubated for 15 minutes at 37°C in a shaking water bath. The reaction was terminated by
the addition of 0.3 ml of ice-cold distilled water followed by vigorous mixing for 5 to 10 seconds. The suspension was rapidly frozen in liquid nitrogen. All cells were disrupted by this treatment, as judged by light microscopy. The amount of cAMP in the lysates was determined using the commercial \[^{125}\text{I}]\text{cAMP kit described above.}

In order to determine the basal levels of intracellular cAMP in different concentrations of cell lysates a commercial kit with detection limits 0.2 to 12.8 pmol cAMP/tube was used. Standards and samples were assayed in replicate tubes. Briefly, 100\(\mu\)l of each sample was incubated with 100\(\mu\)l of \[^{125}\text{I}]\text{cAMP and 100\(\mu\)l antiserum after thorough vortex for 3 hours at 4°C. Then 500\(\mu\)l of Amerlex-M was added and, again after thorough vortex, tubes were incubated for ten minutes at room temperature (15-30°C). Separation of the antibody-bound fraction was achieved by centrifugation for 10 minutes at 1500g. Supernatants were discarded and pellets counted for 60 seconds in a gamma scintillation counter (Pharmacia-LKB, Milton Keynes, U.K.). A typical standard curve and basal levels of intracellular cAMP in different concentrations of PBMCs are presented in Fig 2.6.(A) and (B).

2.2.6. Intracellular cAMP production after stimulation with the \(\beta\)-adrenoceptor agonist, (-)-Isoproterenol.
Since the level of intracellular cAMP in $5 \times 10^5$ cells (a number readily available for our studies) was at the 0.460 pmol level a commercial kit of $[^{125}\text{I}]$ cAMP of detection range 10 to 1600 fmol cAMP/tube was suitable. The standard curve is presented in Fig 2.7. (A). PBMCs from 5 normal subjects were prepared as previously mentioned and $5 \times 10^5$ cells per incubation were suspended in 0.2 ml of buffer containing (-)-isoproterenol of different concentrations ($10^{-3}$ to $10^{-8}$ M). PBMCs were also incubated in the absence of (-)-isoproterenol for the determination of basal levels of intracellular cAMP. Maximum intracellular production of cAMP was observed at the $10^{-4}$ M concentration of (-)-isoproterenol Fig 2.7. (B).

2.2.7. Intracellular production of cAMP after stimulation of PBMCs with Forskolin.

PBMCs $5 \times 10^5$ cells per incubation were stimulated by forskolin at different concentrations (from $10^{-8}$ to $10^{-3}$ M). Forskolin stimulates adenylate cyclase directly and enhances cAMP production at lower concentrations than those of isoproterenol concentrations Fig 2.8.
Fig 2.6. cAMP Assay.

(A) Standard curve. (Each point represents the mean value of three measurements. Intra-assay variation was less than 6%) (B) Total amount of intracellular cAMP from different concentrations of PBMCs (range from $0.5 \times 10^6$ to $4 \times 10^6$ cells per incubation).
Fig 2.7. Intracellular cAMP production after stimulation with Isoproterenol.

(A) Standard curve. (Each point represents the mean value of three measurements. Intra-assay variation was less than 6%) (B) Intracellular production of cAMP after stimulation by (-)-isoproterenol (concentrations from $10^{-8}$ to $10^{-3}$ molar). 1.5 fold increase was observed at concentrations above $10^{-4}$ M isoproterenol (from $980 \pm 50$ fmol at basal state to $1500 \pm 30$ fmol after stimulation with $10^{-3}$ M isoproterenol). cAMP production is expressed in $1 \times 10^6$ cells per incubation.)
Fig 2.8. Intracellular cAMP production after stimulation with Forskolin.

PBMCs from 5 normal subjects were stimulated with different concentrations of Forskolin (10^{-8} to 10^{-3} M). Forskolin at 10^{-5}M stimulated a 3-fold increase from 980±50 at basal state to 3050±45 f mol. cAMP production is expressed in 1X10^6 cells per incubation.
2.2.8. Discussion.

The sensitivity of our cAMP assay was tested by assessing the basal and stimulated levels of intracellular cAMP in intact PBMCs. In basal state, the amount of cAMP was proportional to the number of the incubated PBMCs (Fig 2.6.B). Specific stimulation with the β-agonist (−)-isoproterenol, produced a 1.5 fold increase in cAMP production. This increase in cAMP production was maximum at $10^{-4}$ M isoproterenol concentration.

Further assessment of the sensitivity of our assay, with measurements of intracellular cAMP after direct stimulation of the adenylate cyclase with forskolin, showed a 3 fold increase in cAMP production compared to the basal state. The increase of intracellular cAMP was higher than that observed after β-adrenoceptor stimulation with isoproterenol. Since forskolin has a high affinity for adenylate cyclase, this finding would be anticipated.
2.3. Expression of β-adrenoceptors on PBMCs before and after stimulation with steroids, interleukin-1 and Phytohemagglutinin (PHA).

2.3.1. The effect of steroids on PBMC β-adrenoceptors.

To determine the effect of steroids on the density of β-adrenoceptors on PBMCs, cells from normal subjects were cultured in the presence of different concentrations of hydrocortisone (Fig 2.9.) for 24 hours. Up-regulation of the receptors was observed and it was maximal with $10^{-4}$ M hydrocortisone. 24 hours culture in the presence of hydrocortisone appears to result in the greatest up-regulation of β-adrenoceptors (Fig 2.10.)

2.3.2. The effect of interleukin-1 on PBMC β-adrenoceptors.

The effect of the immune mediator IL-1α upon the density of β-adrenoceptors was assessed by culturing PBMCs from normal subjects in the presence of different concentrations of IL-1α for 24 hours (Fig 2.11.). This treatment resulted in up-regulation of β-adrenoceptors. Maximum up-regulation of receptors was observed after 24 hours (Fig 2.12.)
2.3.3. The effect of mitogenic stimulation with PHA on PBMC β-adrenoceptors.

β-adrenoceptor densities on PBMCs were assessed after activation of the cells by PHA (1μg/ml) for 24 and 48 hours. Enhanced expression of β-adrenoceptors was observed after 24 and 48 hours of incubation reaching its maximum at 24 hours (Fig 2.13.).

2.3.4. Basal and isoproterenol-stimulated intracellular production of cyclic AMP in cultured PBMCs in the presence of hydrocortisone, IL-1α or PHA.

Basal levels of cAMP production by three PBMCs preparations from normal control subjects were similar when cultured in the absence of a mediator and in the presence of hydrocortisone, IL-1α or PHA (1055±24, 1033±37, 1088±52 and 1110±56 fmol/10^6 cells respectively). However, isoproterenol-stimulated levels increased 1.7-fold (1847±197), 2.7-fold (2766±346), 2.6-fold (2811±320) and 3.2-fold (3555±350 fmol/10^6 cells) in the absence of a mediator and in the presence of hydrocortisone, IL-1α and PHA respectively (Fig 2.14.).

2.3.5. Statistical analysis.
The Wilcoxon rank sum test and the paired t-student's test were used. Data are presented as mean±s.e.m. for practical reasons (graphic presentations), despite the occasional
use of nonparametric statistical analysis tests.

**Fig 2.9. β-adrenoceptors on PBMCs after stimulation with Hydrocortisone.**

PBMCs from 3 normal subjects were cultured for 24 hours in the presence of different concentrations of hydrocortisone ($10^{-8}$ to $10^{-3}$ M). Maximum increase of the β-adrenoceptors was observed above $10^{-4}$ M hydrocortisone concentration. (Each point represents the mean value and the bars the s.e.m)
Fig 2.10. Time course development of Hydrocortisone effect

upon β-adrenoceptor expression of PBMCs.

Time course of the development of the hydrocortisone induced increase in β-adrenoceptor density. PBMCs from 3 normal subjects were incubated in the presence of $10^{-4}$M hydrocortisone for the indicated periods of time (2 to 72 hours). The maximum up-regulation of β-adrenoceptors was observed at 24 hours incubation. (Each point represents the mean value and the bars the s.e.m.)
Fig 2.11. Effects of IL-1α upon β-adrenoceptor expression in PBMCs.

PBMCs from 3 normal subjects were incubated for 24 hours in different concentrations of IL-1α. (10^{-13} to 10^{-10} M). Up-regulation of the β-adrenoceptors was observed in the presence of 10^{-12} to 10^{-10} M of interleukin-1α. (Each point represents the mean value and the bars the s.e.m.)
Fig 2.12, Time course of the development of the IL-1α induced increase in β-adrenoceptor density.

PBMCs from 3 normal subjects were incubated in the presence of 20 pM IL-1α for the indicated periods of time, after which β-adrenoceptor densities were assayed. Maximum up-regulation of receptor densities was observed after 24 hours incubation. (Each point represents the mean value and the bars the s.e.m).
Fig 2.13. Effects of PHA upon \(\beta\)-adrenoceptor expression in PBMCs.

PBMCs from 5 normal subjects were cultured with PHA. \(\beta\)-adrenoceptor density before stimulation with PHA was 1343±146 sites/cell and increased to 4090±450 sites/cell after 24 hours culture in the presence of PHA. No further significant increase was observed after 48 hours of incubation (4914±560 sites/cell).*p<0.002 paired t-student test compared to the time 0 values. (Bars: mean±s.e.m.)
Fig 2.14. Intracellular cAMP production in PBMCs stimulated by

**Hydrocortisone, IL-1α and PHA.**

Basal (filled bars) and $10^{-4}$ M isoproterenol-stimulated (crossed bars) intracellular cAMP levels in PBMCs from 3 normal subjects, cultured in the presence of either hydrocortisone or IL-1α for 24 hours. The plus sign indicates $p<0.05$ (Student's t test compared to the media-treated basal levels; the asterisks, $p<0.05$ (Wilcoxon rank sum test) compared to the media-treated stimulated level. (The graphic presentation is in mean±s.e.m despite the use of non-parametric statistical analysis methods)
2.4. Interleukin-2 receptor (IL-2R).

Interleukin 2 (IL-2), formerly called T-cell growth factor, provides a necessary signal for the transition of activated T cells from the G1 to the S phase of the cell cycle (Smith, 1980; Klaus et al., 1984). The specific receptor for IL-2 is a heterodimer consisting of nonconventionally associated p55 and p75 chains with a high affinity (10^{-11} M) for IL-2 (reviewed in Smith, 1989; Waldman, 1991). This receptor is present on activated and, to a lesser extent, resting T cells (Robb and Smith, 1981; Robb et al., 1984). Activated B cells have approximately 10-fold fewer receptors than activated T cells. Natural Killer (NK) cells constitutively express only the p75 chain of the receptor and are unusual in that they lack receptor for antigen and do not require prior sensitisation for expression of the p75 chain of the IL-2R (Dukovich et al., 1987). The IL-2 receptor can be modulated by IL-2 itself as well as TNF and IFNγ (Ruscetti, 1990).

2.4.1. Methods.

2.4.2. High affinity IL-2 receptor (IL-2R) binding on peripheral blood mononuclear cells using ^{125}I Interleukin 2.

It has been suggested that high affinity IL-2
receptors mediate the physiological effects of IL-2 (Robb et al., 1984). To assay these high affinity receptors, we performed radioligand binding studies with $^{125}$I IL-2 (New England, Du Pont de Nemours, Germany) and a specific activity 32 μCi/μg, according to modifications of the method of Robb et al. (1984). PBMCs were isolated as previously described. PBMCs were washed and resuspended in RPMI 1640 medium with 1% albumin and 25 mM HEPES. Total binding was determined by incubating $1 \times 10^5$ cells in a 200μl volume in triplicate with six concentrations (10-150 pM) of $^{125}$I IL-2 for 60 minutes at 37°C. Nonspecific binding was determined over the same concentration range of $^{125}$IIL-2 in the presence of 30 nM unlabelled IL-2 (British Bio-Technology, Oxford, U.K.). After incubation the reaction mixtures were layered onto 400μl ice-cold FCS in a 1.5ml micro-centrifuge tube, and spun at 400xg for 5 minutes. The tips were excised and counted in a LKB minigamma counter (Pharmacia-LKB Milton Keynes, UK) for 60 seconds. Binding kinetics were determined by Scatchard analysis as for the β-adrenoceptor assay. The "specific" binding of $^{125}$I IL-2 rose with increasing $^{125}$I IL-2 concentrations ranging from 10 to 150 pM and amounted to 60% (at 15 pM), 50% (at 31 pM) and 23% (at 150 pM) (Fig 2.15.).

2.4.3. Kinetics for $^{125}$I IL-2 binding.

The time course for binding was determined by
Fig 2.15. Binding Characteristics of $^{125}$I-IL-2.

(A) Binding characteristics of $^{125}$I-IL-2 to PBMCs (1X10^6 cells/ml). The assay was carried out at 37°C for 60 minutes in 96 well plates. Non-specific binding was determined in the presence of 30nM IL2. (Each point represents the mean value of three measurements. The intra-assay variation was less than 6%).
Fig 2.15.

(B) Scatchard analysis revealing a linear plot indicating a single receptor population with $K_D$ of 4.5 pM and $B_{max}$ 49 sites/cell. (Each point represents the mean value of three measurements. The intra-assay variation was less than 6%)
Fig 2.16. Kinetics for $^{125}$I-IL-2 Binding.

PBMCs ($1 \times 10^6$ cells/ml) were incubated at 37°C in round bottom 96 well plates with 75 pM $^{125}$I-IL-2 with and without 30nM unlabelled IL-2. At times indicated the cells were harvested and the mean total and non-specific binding for triplicate wells expressed as counts per minute (CPM). Equilibrium of non-specific displacement was achieved after 20 minutes incubation. (Each point represents the mean value of three measurements. The intra-assay variation was less than 6%).
incubating 1 x 10^5 cells with 75 pM \( ^{125}\text{I} \) IL-2 in the presence and absence of 30nM IL-2 at 37°C. Specific binding was determined in triplicate at various times after the addition of \( ^{125}\text{I} \) IL-2 and expressed as CPM (Fig 2.16.)

### 2.4.4. Phytohemaglutinin (PHA)-induced lymphocyte proliferation.

PBMCs were incubated in a 96-well microtiter plate (Titertek) in triplicate containing 1x10^5 cells in a total volume of 200μl of RPMI 1640 medium plus 10% FCS, 100IU/ml of penicillin, 100μg/ml streptomycin, and 1μg/ml PHA. Cells cultured in the absence of PHA acted as negative controls. [\(^3\text{H}\)]thymidine (1μCi) was added in each well at different times of incubation. After an additional 6 hours in culture, the cells were harvested using a Titertek cell harvester on filter mats (Skatron, Newmarket, UK). The mats were dried and radioactivity was evaluated with a liquid scintillation counter. Maximum proliferation was observed after 48 incubation (Fig 2.17).

### 2.4.5. High affinity IL-2R densities on PBMCs stimulated with PHA.

High affinity IL-2Rs on PBMCs were assessed after activation of the cells by PHA (1μg/ml) for 24 and 48 hours. Enhanced expression of IL-2Rs was observed on both occasions with a higher expression at 48 hours. The
affinity of the receptors did not significantly change in comparison with those on resting PBMCs (Fig 2.18., Fig 2.19.).

2.4.6. Results.

IL-2R expression on resting and stimulated (48 hours with PHA) PBMCs was assessed in 25 healthy subjects aged 23 to 60 years old. The mean±sem values are presented in Table 2.2.

Table 2.2. IL-2R expression on resting and PHA-stimulated PBMCs. The density of the receptors (B\text{max}) is expressed in sites/cell and affinity (K\text{D}) is expressed in pM.

<table>
<thead>
<tr>
<th></th>
<th>Basal state</th>
<th>After 48 hours stimulation with PHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>B\text{max} (sites/cell)</td>
<td>50±4</td>
<td>1490±56</td>
</tr>
<tr>
<td>K\text{D} in pM</td>
<td>4±0.8</td>
<td>6±0.3</td>
</tr>
</tbody>
</table>

2.4.7. Discussion

The binding assay of IL-2Rs on resting PBMCs and within the range of concentrations 10 to 150 pM $^{125}$ I-IL-2, appeared to be saturable. The Scatchard analysis indicated that the receptors are of homogeneous origin with the same affinity. Kinetic studies showed that a binding equilibrium was achieved within 20 minutes of incubation. Both the values of B\text{max} expressed as sites/cell and the
Fig 2.17. Proliferation rate after stimulation with PHA.

Maximum proliferation was observed after 48 hours culture in the presence of PHA (150±50) compared to the basal state value which was considered equal to 1 (Logarithmic expression, bars; means±s.e.m.).
Fig 2.18. IL-2R expression after stimulation with PHA.

High affinity IL-2R expression on PBMCs from 5 normal subjects stimulated with PHA for 24 and 48 hours. At rest, the IL-2R density is 50±4 sites/cell and after incubation for 24 hours there is 530±20 sites/cell and 48 hours there is 1443±50 sites/cell with K_D values of 5±0.5pM and 6±2 pM respectively (Bars; mean±s.e.m.).
Fig 2.19. Binding Characteristics for IL-2Rs after stimulation with PHA.

(A) Binding characteristics of $^{125}$I-IL-2 to PHA-stimulated (48 hours) PBMCs. (Each point represents the mean value of three measurements. The intra-assay variation was less than 6%).
Fig 2.19.

(B). Scatchard analysis indicating a population of IL-2Rs with $K_D$ 5.8 pM and $B_{max}$ 1480 sites/cell.
affinity values expressed in pM are compatible with those reported by Robb et al. (1984). Stimulation of PBMCs with PHA led to an enhanced expression of IL-2Rs reaching its maximum after 48 hours. The affinity of IL-2Rs on activated PBMCs did not differ from that on resting PBMCs. By using a binding assay, we have been able to measure high affinity receptors, while other methods such as anti-Tac fluometry, identifies both low and high affinity receptors. The low-affinity binding sites of IL-2 are unlikely to directly participate in normal physiological responses, since at concentrations that might occur in vivo (<200pM) (Robb et al., 1984) only a minute fraction (<1%) of such sites would be occupied. In contrast, the same concentration would saturate the high affinity sites. Since the binding assay identifies the receptors on living cells, expression of IL-2Rs under various in vitro influences can also be investigated.
2.5. Regulation of proliferation rate and expression of IL-2Rs through β-adrenoceptor stimulation.

2.5.1. Regulation of high affinity IL-2R expression on PBMCs by stimulating β-adrenoceptors.

The potential regulatory role of β-adrenoceptors in activated lymphocytes has been emphasized by the demonstration of suppression of IL-2R following β-adrenergic stimulation (Fildmen et al., 1987). It has been indicated that this action is mediated through cAMP (Johnson and Smith 1990). To determine if such an effect occurred with PBMCs, we cultured PBMCs from normal subjects for 48 hours (maximum expression of IL-2Rs) in the presence of a range of different concentrations of β-agonist isoproterenol (10^{-8} to 10^{-4} M) and 1mM Ascorbic acid. Suppression of IL-2R expression was observed with isoproterenol concentrations above 10^{-5}M (Fig 2.20.)

2.5.2. Proliferation rate of stimulated by PHA PBMCs in the presence of the β-agonist Isoproterenol.

PBMCs (1x10^5) were incubated in triplicate in a 96-well microtiter plate (Titertek) in a total volume of 200μl of RPMI 1640 medium plus 10% FCS, 100IU/ml of penicillin, 100μg/ml streptomycin, and 1μg/ml PHA. Cells were cultured in the presence of isoproterenol at concentrations ranged from 10^{-8} to 10^{-4} M. [^{3}H]thymidine
incorporation was assessed, as previously described, after a 48 hours incubation. As a control were cells cultured in the absence of PHA. Suppression of the proliferation rate was observed at $10^{-6}$ to $10^{-4}$M isoproterenol concentrations (Fig 2.21.).

2.5.3. Regulation of high affinity IL-2Rs on activated PBMCs by β-agonist or hydrocortisone.

Activated PBMCs (stimulated with PHA for 48 hours) PBMCs washed twice and cultured for another 48 hours in the presence of $10^{-6}$ M Isoproterenol or $10^{-4}$M hydrocortisone (a concentration which caused a 2 fold increase in β-adrenoceptor density). Suppression of IL-2Rs was observed in the presence of Isoproterenol but not hydrocortisone (Fig 2.22.)

2.5.4. Discussion.

β-adrenoceptor stimulation leads to increase intracellular cAMP. cAMP appears to be involved in regulation of both proliferation and IL-2R expression. The activation and proliferation of T cells can be resolved into two distinct phases; the transition from resting cells (G₀) to the early G₁ stage of the cell cycle by T cell receptor (TCR) stimulation, followed by IL-2-mediated G₁ progression and S phase transition. Both of these phases are inhibited by elevated cAMP. A number of studies have
Fig 2.20. IL-2 receptor expression on PBMCs after stimulation with PHA and Isoproterenol.

PBMCs from 5 normal subjects were cultured in the presence of PHA and various concentrations of the β-adrenergic agonist Isoproterenol (10⁻⁸ to 10⁻⁴ M). At 10⁻⁴ M Isoproterenol the IL-2R expression was suppressed by 50% (from 1440±60 sites/cell to 630±29 sites/cell). *p<0.02; paired t-student test (Bars; mean±s.e.m.)
Fig 2.21. Proliferation Rate of PBMCs after stimulation with PHA and Isoproterenol.

Proliferation rate was assessed after 48 hours incubation in the presence of PHA and different concentrations of the β-adrenergic agonist Isoproterenol (10^-5 to 10^-4 M). Suppression of proliferation (with a maximum of 40%) was observed with concentrations of isoproterenol above 10^-6 M (from 150±50 to 81±25 units). The rate has been expressed in units where one unit is equal to the basal (untreated) proliferation rate. (*p<0.02 paired t-students test, Bars: mean±s.e.m.)
Fig 2.22. IL-2Rs on PBMCs after stimulation with Hydrocortisone or Isoproterenol.

PBMCs from three normal subjects were stimulated for 48 hours with PHA (1 μg/ml) and then further cultured in the presence or absence of isoproterenol 10^{-4} M or hydrocortisone 10^{-4} M. The expression of high affinity IL-2Rs in control cultures was 863±148 sites/cell. In the presence of isoproterenol there were 327±43 sites/cell and in the presence of hydrocortisone, 1040±106 sites/cell. Suppression of IL-2Rs was observed only in the presence of isoproterenol (*p<0.02 Wilcoxon rank sum test. Graphic presentation with bars of means±s.e.m.).
shown that the elevation of cellular cAMP inhibits several events associated with TCR activation, including the generation of inositol phosphates, calcium mobilization, protein phosphorylation, IL-2 production, and IL-2R expression (Saltzman et al., 1989; Asao et al., 1990; Merida et al., 1990; Klausner et al., 1987; Mary et al., 1989). Inasmuch as our studies indicate that high affinity IL-2R expression and proliferation rate of PBMCs are decreased after stimulation of β-adrenoceptors, this may have significant implications for the regulation of the in vivo immune response. In vitro, the β-agonist isoproterenol, at concentrations of 10^{-6}M and 10^{-4}M could suppress proliferation rate of PBMCs (by 40%) and expression of high affinity IL-2Rs (by 50%) respectively. Compatible results have been reported by previous investigators (Johnson and Smith, 1990). Since suppression of proliferation and IL-2Rs was achieved at different concentrations of Isoprotenerol, probably different mechanisms are involved in these processes as was suggested by Gaulton and Eardley (1986). The suppression of high affinity IL-2Rs has been specifically attributed to the active phosphorylation of the 75 KDa subunit, possibly involving the protein kinase A. Active suppression of IL-2Rs through β-adrenoceptor stimulation was further supported by our in vitro studies which demonstrated that isoproterenol, but not hydrocortisone, could suppress IL-2R expression in PHA-activated PBMCs.
A significant drawback for any therapeutic application of our observations could be the requirement of isoproterenol concentrations which would not be tolerated in vivo. However, it may be the case that lower concentrations of a $\beta$-agonist in vivo could have a significant impact on the immune responses.
CHAPTER 3

EXPRESSION OF $\beta$-ADRENOCEPTORS AND IL-2 RECEPTORS ON PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCs) IN MULTIPLE SCLEROSIS.

3.1. $\beta$-adrenoceptor densities on PBMCs in MS patients: a regulatory role for cortisol and interleukins?

Pathologically multiple sclerosis (MS) is classified as an inflammatory demyelinating disease of the central nervous system (CNS) (Allen et al., 1992). The aetiology remains unknown, but evidence exists to support the hypothesis of environmental agent or agents acting in a genetically susceptible population, to induce the disease. After a variable latent period, the disease manifests with episodes of immune-mediated focal demyelination of the CNS. Classically MS begins as a relapsing remitting disease. As the disease progresses remission may be less complete and the patient may pass into a secondary progressive phase. A minority of patients (approximately 8%), present with a primary progressive course and approximately 25% of patients remain well 10 to 15 years after the onset of the disease, with so called benign MS (Weinshenker et al., 1989). The variations in the clinical presentation and the course of the disease remain unexplained. Reduced disease activity or activation of recovery mechanisms which limit the inflammatory process
may give as a result the benign form of the disease. In this context immunoregulatory mechanisms may contribute, involving interactions among the immune, neural, and endocrine systems.

Important observations regarding the role of neural and endocrine parameters, known to exert feedback control on immunological reactivity, have been observed during the preclinical and clinical stages of both primary acute and adoptively transferred experimental autoimmune encephalomyelitis (EAE) which is the animal model for MS. An increased density of $\beta$-adrenoceptors on splenic lymphocytes has been observed in EAE when immunoproliferative responses are most marked (MacKenzie et al., 1989; Leonard et al., 1990). As lymphocyte functions are altered by stimulation of $\beta$-adrenoceptors (Felten et al., 1987), which are themselves regulated by a range of cytokines and growth factors (Nakane et al., 1990), the assessment of density of $\beta$-adrenoceptors on circulating lymphocytes in conjunction with measuring plasma cortisol levels, in patients with different clinical categories of MS, other inflammatory and non inflammatory autoimmune diseases as rheumatoid arthritis (RA) and myasthenia gravis (MG) could define the importance of $\beta$-adrenoceptors on PBMCs in relation to disease activity or the recovery phase.

3.1.1. Methods.
3.1.2. Subjects.

In this study, were included 33 patients with MS diagnosed according to the standard criteria of Poser and associates (1983). β-Adrenoceptor density on PBMCs was assessed blindly in all patients. Subsequently, the patients were divided into groups: 13 patients with secondary progressive MS, aged 26 to 55 years old, 14 patients with relapsing-remitting MS, aged 23 to 45 years old (8 in relapse and 6 in remission), and 6 patients with benign MS, aged 38 to 45 years old (2 in relapse and 4 in remission).

Clinical and magnetic resonance imaging (MRI) criteria were used to define relapse and remission. Clinically relapse was defined as the occurrence of a symptom or symptoms of neurological dysfunction, with or without objective confirmation lasting more than 24 hours, and remission was defined as a definite improvement in signs or symptoms, lasting for at least 1 month (Poser et al., 1983). By MRI criteria, relapse and remission were defined by the presence or absence of gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA)-enhanced lesions. The general diagnostic criteria for the grouping are shown in Table 3.1. All patients were assessed by the expanded disability status scale (EDSS) of Kurtzke (Kurtzke, 1983) and none had a disability score higher than 7. Sixteen volunteers, matched by age and sex, were
tested as normal control subjects. Eleven patients with various noninflammatory neurological diseases (4 with Parkinson's disease, 3 with epilepsy, 2 with a brain tumour, and 2 with chronic back pain) were included, as were 14 patients with the chronic active form of rheumatoid arthritis (RA), aged 19 to 78 years old, and 5 patients with myasthenia gravis (MG), aged 25 to 40 years old, a comparative group with a noninflammatory autoimmune disorder. None of the patients with MS had received prior immunosuppressive treatment and none had received corticotropin or glucocorticoid treatment in the preceding 6 months. All the patients with RA had been treated with penicillamine and the patients with MG had received anticholinesterase treatment for the previous 2 years. The MS patients were selected and clinically assessed by Dr Y. Zoukos. RA patients were assessed by Dr D. Scott St. Bartholomew's Hospital. The studies were carried out having the ethical approval of the National Hospital for Neurology and Neurosurgery Ethics Committee.

3.1.3. Isolation of PBMCs and $\beta$-adrenoceptor assay.

Isolation of PBMCs and $\beta$-adrenoceptor assay was carried out according to the methods described previously. To determine the exact contribution of lymphocytes and monocytes to the total binding sites in our assays, monocytes were depleted by adherence to plastic in RPMI 1640 (Gibco, Uxbridge, UK) containing 5% fetal calf serum
(FCS) (ICN Biomedicals Ltd, High Wycombe, UK) 100 IU/ml of penicillin, and 100 μg/ml of streptomycin (Gibco), for 1 hour at 37° in 95% air and 5% carbon dioxide. The β-adrenoceptor assay was carried out on non-adherent lymphocytes.

Table 3.1. Clinical Criteria for Classification of Patients with MS (Thompson et al. 1991)

<table>
<thead>
<tr>
<th>Classification</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secondary Progressive</td>
<td>Patients with a relapsing-remitting form of the disease who have entered a progressive phase, losing during the last year at least 1 point of the Kurtzke disability scale without a clinical relapse.</td>
</tr>
<tr>
<td>Relapsing-remitting</td>
<td>Patients with a relapsing-remitting form of the disease and with at least three clinical relapses during the last two years.</td>
</tr>
<tr>
<td>Benign</td>
<td>Patients with a relapsing-remitting form of the disease and duration more than ten years, and disability score less than 3 of the Kurtzke scale.</td>
</tr>
</tbody>
</table>

3.1.4. Plasma cortisol determination.

Plasma from the heparinized blood samples was aliquoted and stored at -20°C. Plasma samples were thawed and assayed for cortisol using ¹²⁵I-labelled cortisol radioimmunoassay kit (Immunodiagnostics, Tyne and Wear, UK). This is a coated tube assay, utilising anti-cortisol IgG bound to the inner surface of a polypropylene tube and ¹²⁵I-labelled cortisol. The sample is incubated with ¹²⁵I-
cortisol for 45 minutes at 37°C in the antibody-coated tubes. After incubation the tubes are decanted to remove unbound \(^{125}\text{I}\)-cortisol and then counted, the bound radioactivity being inversely proportional to the

\[\begin{array}{cccccc}
\text{nmol/lit cortisol} & 50 & 100 & 200 & 400 & 800 & 1600 \\
\%	ext{Percent of tracer binding B/Bo} & 90 & 80 & 70 & 60 & 50 & 40 \\
\end{array}\]

**Fig 3.1. Cortisol Assay.**

Standard curve for indicated quantities of cortisol used to calculate sample levels of cortisol (Each point represents the mean value of three measurements. The intra-assay variation was 6%).
concentration of cortisol. The standard curve from which cortisol levels are calculated is presented in Fig 3.1. Cortisol assays were carried out in triplicate and the mean value for each sample was calculated.

3.1.5. Measurement of intracellular cAMP.

For the measurement of basal and stimulated intracellular cAMP levels, PBMCs $5 \times 10^5$ per well in 96 well plates incubated in the presence of $10^{-6}$ M isoprotenerol, the concentration producing maximal intracellular cAMP production. cAMP assay was carried out as previously described.

3.1.6. Statistical analysis.

Data are presented as mean ± standard error of the mean (SEM). The Wilcoxon rank sum test was used for the analysis of the densities of β-adrenoceptors on circulating lymphocytes, the plasma cortisol levels and the cAMP levels. Student's t test was used for paired samples. Statistical significance was accepted at a 95% confidence level of two tailed (p<0.05).

3.1.7. Results.

3.1.8. β-adrenoceptor density on PBMCs.

The results of the radioligand-binding assay for β-adrenoceptors are presented in Table 3.2. The receptor
binding assays were saturable, stereospecific and competitive in PBMCs from all clinical groups. The affinity of the receptors $K_p$ was 128±12 pM in normal control subjects, with $B_{\text{max}}$ of 1227±41 sites/cell. A statistically significant increase in the density of these receptors was observed in patients with secondary progressive MS and those in relapse and also in patients with RA, compared to normal control subjects. In the patients with benign MS, the density of the receptors was within the normal range, although in 2 patients in relapse, a high receptor density was observed (1806 and 2690 sites/cell). In patients with MG and the other neurological control subjects, the densities were within the normal range. $K_p$ and $B_{\text{max}}$ of 5 control and 5 MS monocyte-depleted preparations did not differ from those determined in the non-depleted sample assay. The adherent cell population (monocytes) from 10 control subjects had similar binding and affinity sites to the non-adherent cells ($\beta$-adrenoceptors: 1350±120 sites/cell and $K_p$=110±20 pM). Therefore, the presence of monocytes in the samples would not be expected to influence the overall findings.

3.1.9. Basal and isoproterenol-stimulated intracellular production of cAMP.

Basal and (-)-isoproterenol-stimulated production of cAMP was assessed in PBMCs from 5 patients with MS with a high density of receptors and from the 5 normal subjects.
Mean basal cAMP in the PBMCs from the normal control subjects and MS patients did not differ significantly (970±39 versus 1100±44 fmol/10^6 cells), respectively. After stimulation cAMP production increased 1.5 fold in PBMCs from normal controls to 1469±54 fmol/10^6 cells, and 2.3-fold in MS patients to 2544±101 fmol/10^6 cells Fig 3.2.

3.1.10. Plasma levels of cortisol.

Cortisol levels were assessed in samples collected at 10 A.M. In the group of patients with relapsing-remitting MS, patients who were in relapse had significantly high plasma cortisol levels compared to normal control levels. However, no statistically significant differences were observed between plasma cortisol levels in the other patients and those in normal control subjects (Table 3.2.).

3.1.11. The effect of hydrocortisone and interleukin-1 on PBMC β-adrenoceptors from normal subjects and secondary progressive MS patients.

The effect of steroids and cytokines upon the density of β-adrenoceptors on PBMCs, from a separate group of 5 patients with secondary progressive MS (all with a high density of receptors) and 5 normal control subjects, was assessed in vitro by culturing cells in the presence and
Table 3.2. Peripheral blood mononuclear cell receptor density and plasma cortisol in MS, normal controls, and other diseases.

<table>
<thead>
<tr>
<th>Test Groups</th>
<th>No of subjects</th>
<th>$K_D$ (pM)</th>
<th>Bmax (sites/cell)</th>
<th>Plasma cortisol (mmol/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal controls</td>
<td>16</td>
<td>128±12</td>
<td>1227±41</td>
<td>353±37</td>
</tr>
<tr>
<td>Multiple sclerosis</td>
<td>33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary progressive</td>
<td>13</td>
<td>170±45</td>
<td>2125±81$^a$</td>
<td>371±36</td>
</tr>
<tr>
<td>Relapsing</td>
<td>8</td>
<td>99±8</td>
<td>2087±254$^b$</td>
<td>728±149$^b$</td>
</tr>
<tr>
<td>Remitting</td>
<td>6</td>
<td>102±9</td>
<td>1384±175</td>
<td>487±73</td>
</tr>
<tr>
<td>Benign</td>
<td>6</td>
<td>174±48</td>
<td>1384±836</td>
<td>498±146</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>14</td>
<td>148±39</td>
<td>2289±131$^a$</td>
<td>451±60</td>
</tr>
<tr>
<td>Myasthenia gravis</td>
<td>5</td>
<td>122±14</td>
<td>1372±74</td>
<td></td>
</tr>
<tr>
<td>Neurological controls$^c$</td>
<td>12</td>
<td>94±58</td>
<td>943±396</td>
<td>401±66</td>
</tr>
</tbody>
</table>

$^a p<0.01, ^b p<0.05$ Wilcoxon rank sum test statistical values in comparison with normal controls

$^c$Includes patients with Parkinson's disease (4), epilepsy (3), brain tumours (2), and chronic pain (2).
Fig 3.2. Intracellular cAMP production in PBMCs from MS patients.

Basal and $10^{-4}$ M isoproterenol-stimulated intracellular cAMP production in peripheral blood mononuclear cells from 5 normal subjects (open bars) and 5 patients with secondary progressive MS and high densities of $\beta$-adrenoceptors (2342±80 sites/cell) (crossed bars). The asterisk indicates $p<0.05$ compared to MS basal levels and normal control stimulated levels; the plus sign, $p<0.05$ compared to normal control basal levels (Wilcoxon rank sum test). The graphic presentation is in mean±s.e.m despite the use of non-parametric statistical analysis test.
Fig 3.3. β-adrenoceptors on PBMCs from MS patients after stimulation with Hydrocortisone or Interleukin-1α.

PBMCs from 5 normal subjects (open bars) and 5 patients with secondary progressive MS with high densities of β-adrenoceptors (crossed bars) were cultured in media and in the presence of hydrocortisone or IL-1α for 24 hours. A twofold increase in the density of β-adrenoceptors was observed in the PBMCs from normal subjects in the presence of either hydrocortisone or IL-1α, but not in the PBMCs from the patients with MS. The asterisks indicate p<0.05 (Wilcoxon rank sum test). The graphic presentation is in mean±s.e.m. despite the use of non-parametric statistical analysis test.
absence of hydrocortisone (10^{-4} M) and IL-1α (20 pM) for 24 hours. At these concentrations in vitro, PBMC β-adrenoceptor density was up-regulated to the same extent as to that observed in MS patients.

The density of β-adrenoceptors in PBMCs from the 5 normal control subjects increased more than twofold in the presence of hydrocortisone or IL-1α, while in the PBMCs from the 5 MS patients, no significant increase was observed in the presence of either mediator (Fig 3.3.).

3.1.12. Discussion.

In this study, a twofold increase in the density of β-adrenoceptors in PBMCs was demonstrated in patients with secondary progressive or relapsing MS. Also this study extends previous observations in progressive MS (Karaszewski et al., 1990) to include patients with benign and those with relapsing-remitting MS in which a positive correlation between disease activity and receptor density was apparent (Fig 3.4.). Also included in this study were patients with RA, which is an autoimmune inflammatory disorder, and patients with MG, an autoimmune disorder without inflammatory characteristics. The clear-cut differences in β-adrenoceptor densities found in the inflammatory conditions emphasize the possible link with the immunological process. Hypersensitivity up-regulation of β-adrenoceptors has been observed in patients with pure
autonomic failure characterized by orthostatic hypotension (Bannister et al., 1981; Hui et al., 1981) and autonomic dysfunction in MS patients could contribute to the observed β-adrenoceptor up-regulation. However, none of the studied RA and MS patients had overt clinical signs of autonomic dysfunction.

In PBMCs from normal control subjects, a 1.5-fold increase in cAMP production following isoproterenol stimulation is consistent with previous observations, considering the mean age of the tested individuals (Halper et al., 1984). In the patients with MS, the basal levels of cAMP were no different from those of normal control subjects, but the greater than twofold increase of cAMP production after isoproterenol stimulation mirrors the differences in the receptor densities, indicating that this up-regulation is functional.

In MS patients lymphocytic subpopulation alterations have been reported during the active phase of the disease with a decrease in CD8⁺ lymphocytes (Compston, 1983). Since different lymphocytic subpopulations exhibit different numbers of β-adrenoceptors such alterations could be accountable for our observations in MS. CD8⁺ lymphocytes possess the highest densities of β-adrenoceptors and therefore it would be expected that a decrease in the number of CD8⁺ cells in MS would result in an overall decrease of β-adrenoceptors rather than the observed
Fig 3.4. β-adrenoceptors and clinical status.

β-Adrenoceptor densities on PBMCs in relation to clinical status: relapse and remission. Six patients were in remission and had no gadolinium-enhanced lesions on magnetic resonance imaging (MRI) (filled bar); the mean receptor value was 1384±175 sites/cell. Eight patients were in relapse (7 in clinical relapse with MRI findings and only 1 with new MRI lesions, crossed bar), with a mean receptor value of 2087±254 sites/cell. The asterisk indicates p<0.05 (Wilcoxon rank sum test). Graphic presentation is in mean±s.e.m. despite the use of non-parametric statistical analysis test.
increase. In the study of Karaszewski et al. (1990, 1991), it was shown that although increase of β-adrenoceptors occurred in all lymphocytic subpopulations this was more marked in CD8+ cells.

In both actively induced and adoptively transferred EAE in Lewis rats, the increased β-adrenoceptor density observed on splenic lymphocytes is considered to mark the expansion of antigen-specific cells which, at least in vitro, results in enhanced expression of β-adrenoceptors (Felten et al., 1987). The comparable phenomenon in MS could result from persisting immune reactivity and consequent cytokine production. Both hydrocortisone and IL-1α have been reported to have an up-regulatory effect on β-adrenoceptors in a number of cell populations (Marone et al., 1980) and to potentiate the isoproterenol-induced increase in cAMP accumulation in cultured human lung tumour cells (Nakane et al., 1990). Our in vitro studies showed a twofold increase in the density of receptors on PBMCs from normal control subjects in the presence of either hydrocortisone or IL-1α, while no further change was observed in PBMCs of the MS group, which already had increased receptor density. The increase in cAMP production in cultured control lymphocytes indicates a functional up-regulation similar to that already present in many patients with MS. Does this in vitro up-regulation of β-adrenoceptors on PBMCs by soluble inflammatory mediators reflect events that are taking place in vivo in
patients with MS and RA? Although plasma cortisol levels in the patients with progressive MS were not significantly greater than normal values, in the MS patients who were in relapse, increases reached a 0.02 degree of significance. Previous studies failed to demonstrate a persistent increase in plasma cortisol levels in patients with MS, but increases were observed during clinical relapse (Millac et al., 1969; Teasdale et al., 1967). Cerebrospinal fluid and plasma levels of cytokines in patients with MS and their relation to disease activity have recently received much attention. Although the findings were inconsistent, there was some evidence of increases in plasma cytokine levels in MS and an increased production of IL-1 and IL-2 by mononuclear cells of MS patients in vitro (Brajczwaska-Fischer et al., 1988; Merril et al., 1988). In patients with RA, raised plasma levels of IL-1 were also observed (Eastgate et al., 1988). The role of cAMP in immune activity was extensively studied in vitro and an immunosuppressive action postulated (Kammer, 1988). More recently, a direct inhibition of T-cell mitogenesis and of IL-2 receptor expression was attributed to cAMP (Krause et al., 1991).

It has been suggested that the up-regulation of lymphocyte β-receptors in progressive MS may be the result of denervation hypersensitivity of the sympathetic nervous system (Karaszewski et al., 1990; Karaszewski et al., 1991) and this hypothesis should be further tested by a
thorough autonomic function study in MS patients. However, the up-regulation of receptors in RA suggests that an explanation may lie in the inflammatory process itself; possibly the phenomenon is a characteristic of chronic inflammatory disease in general. Whether this is a recovery mechanism in response to inflammation, requires further investigation in a longitudinal study of MS patients. Alternatively, it may be acting to blunt homeostatic immunoregulatory processes. The evidence for the modulation of EAE by β-adrenoceptor agonists (Chelmicka-Schorr et al., 1989) emphasizes the possible role of these receptors in regulating inflammation in immune-mediated disease.
3.2. Expression of high affinity IL-2Rs and \( \beta \)-adrenoceptors on PBMCs: correlations with clinical and MRI activity in relapsing remitting MS patients.

The increase in \( \beta \)-adrenoceptor density on PBMCs from MS patients can be attributed to either sympathetic dysfunction, inflammation or to a combination of the two (Karaszewski et al., 1990; Zoukos et al., 1992a). Although there is evidence of autonomic dysfunction involving predominantly the central autonomic nervous system in progressive MS, as in multiple system atrophy (MSA) (Thomaides et al., 1993b), the normal \( \beta \)-adrenoceptor density on PBMCs in the latter group (Zoukos et al., 1993) probably rules this out as an explanation for the increased expression in MS. A link between high densities of \( \beta \)-adrenoceptors and inflammatory disease was indicated in the study of rheumatoid arthritis (RA) and relapsing-remitting MS patients (Zoukos et al., 1992a). Certainly, enhanced expression of \( \beta \)-adrenoceptors on PBMCs can be stimulated in vitro by the inflammatory mediators, cortisol, IL-1 and IL-2 (Zoukos et al., 1992a, Karaszewski et al., 1991).

A potential regulatory role of \( \beta \)-adrenoceptors has been emphasized by demonstrating inhibition of IL-2R expression on activated lymphocytes after \( \beta \)-adrenoceptor stimulation (Feldman et al., 1987). Although there have been reports of increased soluble IL-2R expression during
the active phase of the disease in serum and cerebrospinal fluid (CSF) of MS patients (Adachi et al., 1990; Kittur et al., 1990; Sharief et al., 1991), this has not been confirmed in a serial study (Freedman et al., 1992).

To investigate further the possible association of β-adrenoceptor density alterations with disease activity and with immune parameters, a six month longitudinal study of relapsing-remitting patients was designed. β-adrenoceptor and IL-2R densities on PBMCs and plasma levels of cytokines were assessed monthly while disease activity was monitored clinically and by magnetic resonance imaging (MRI). MRI has been chosen as it appears to be highly sensitive in recording disease activity particularly with the addition of the contrast agent Gadolinium-DTPA which indicates blood-brain barrier breakdown in association with inflammation (Kermode et al., 1990; Miller et al., 1988; Hawkins et al. 1990). Furthermore, appropriate in vitro experiments were also designed to elucidate the influence of β-adrenoceptor stimulation upon expression of IL-2Rs and proliferation of lymphocytes.

3.2.1. Methods.

3.2.2. Subjects.

In the six month follow-up study (with monthly assessments) 12 MS patients were included who were diagnosed according to the standard criteria of Poser and
associates (1983). Six patients had the benign form of MS with a relapsing-remitting course of more than 10 years and a Kurtzke expanded disability status score (EDSS) of three or less (Kurtzke, 1983). The other six patients had a relapsing-remitting form of the disease and a history of less than 5 years and a record of at least 2 relapses during the last two years. The range of age for the benign patients was 36 to 54 years old (mean 47 years) and for the relapsing-remitting 27 to 45 years old (mean 34 years). All patients were followed up at monthly intervals by Dr D. Kidd when they were questioned about new or recurring symptoms and reexamined. Clinical criteria were used to define relapse and remission; relapse was defined as the occurrence of a symptom or symptoms of neurological dysfunction, with or without objective confirmation, lasting more than 24 hours, and remission was defined as a definite improvement in signs or symptoms, lasting for at least 1 month (Poser et al., 1983). None of the patients had received immunosuppressive treatment prior to the study and none had received steroid treatment during the study period. Six normal, age and sex matched controls (NC) were also studied serially; 4 female and 2 male, aged 27 to 55 years old (mean 40 years). The studies were approved by the Ethics Committee of the National Hospital for Neurology and Neurosurgery, Queen Square.

3.2.3. Magnetic Resonance Imaging (MRI).
All patients underwent MRI scan of brain with Gadolinium enhancement each month. Scans were performed on a Signa 1.5T superconducting system (GE Milwaukee). T₂ weighted and proton density weighted images were acquired with 34 axial slices parallel to a plane between the anterior and posterior limbs of the corpus callosum using a VEMP spin echo sequence TR 2500 msec, TE 80 msec and TE 32 msec respectively. Five minutes following injection of 0.1 mmol/kg Gadolinium-DTPA (Magnevist⁸) a T₁ weighted sequence TR 500 msec TE 19 msec was acquired. Images were plotted in a 256 x 192 image matrix. The scans were examined by a neuroradiologist (Dr B.E. Kendall) who was blinded to the clinical details. Lesions were counted in five periventricular and eight non-periventricular white matter regions and added, and lesions were measured according to their longest diameter as 1:<5mm, 2:5-10mm, 3:>10mm and 4:confluent. Lesion load was measured by adding the lesion scores. The occurrence of new and enlarging lesions with or without enhancement was noted over time (T₁ and T₂ weighted images from patient 5; Table 3.3 and patient 4; Table 3.4 are presented in Appendix (page: 273-287).

3.2.4. β-adrenergic receptor assay.

PBMCs were washed in PBS and resuspended at a concentration of 5x10⁶ cells/ml. A ligand-binding assay with [¹²⁵I] iodocyanopindolol (CYP) (Amerham, Aylesbury, UK), specific activity 2000 μCi/mmol, was used, as
3.2.5. Interleukin 2 receptor assay.

Two populations of IL-2Rs have been characterized according to their affinity for IL-2 in radioligand binding studies, i.e. high affinity and low affinity IL-2Rs (Robb et al., 1984, Robb et al., 1985). It has been demonstrated that it is the high affinity IL-2Rs which mediate the physiologic effects of IL-2 (Robb et al., 1984). The high affinity sites were measured with a radioligand binding assay using $[^{125}\text{I}]$ IL-2 (New England Nuclear, Du Pont de Nemours, Germany) specific activity $32\mu\text{Ci}/\mu\text{g}$, according to modifications of the method of Robb et al. (1984, 1985) as previously described (Chapter 2).

3.2.6. IL-2R densities on PBMCs after stimulation with PHA in the presence and absence of isoproterenol.

IL-2R densities on PBMCs were assessed after stimulation with PHA (1μg/ml) in the presence and absence of isoproterenol (10^{-4}\text{M}) for 48 hours.

3.2.7. Phytohemaglutinin (PHA)-induced lymphocyte proliferation in the presence and absence of isoproterenol.

PBMCs were incubated in a 96-well microtiter plate
(Titertek) in triplicate containing $1 \times 10^5$ cells in a total volume of 200μl of RPMI 1640 medium plus 10% FCS, 100 IU/ml of penicillin, 100μg/ml streptomycin, and 1μg/ml PHA. Cells cultured in the absence of PHA were used as controls. To assess the effect of isoproterenol upon proliferation, cells at the same concentration were cultured in the presence of $10^{-5}$M isoproterenol (Sigma, UK) and 1mM ascorbic acid. Suppression in proliferation of PBMCs has been observed after stimulation with isoproterenol above $10^{-6}$M concentration (Chapter 2). At $10^{-5}$M concentration, the proliferation rate was 60% of the unstimulated cells. After 48 hours (maximum proliferation) $[^3H]$thymidine (1μCi) was added to each well. After an additional 6 hours in culture, the cells were harvested using a Titertek cell harvester on filter mats (Skartron, Newmarket, UK). The mats were dried and radioactivity was evaluated with a liquid scintillation counter.

All assays were carried out by Dr Y. Zoukos, blind to clinical information and the results were analysed subsequently.

3.2.8. Statistical analysis.

Data, where appropriate are presented as mean ± sem. The Wilcoxon rank sum test was used for the analysis of the differences in densities of β-adrenoceptor and IL-2R. The Student's t-test was used for paired samples. The probabilities of coexistence of clinical or MRI activity
with a high expression of IL2-R or β-adrenoceptor on PBMCs was analyzed by χ² test or Fisher's exact test. Statistical significance was accepted at a 95% confidence level (p<0.05).

3.2.9. Results.

All data are presented in Table 3.3 and 3.4. There was a total of four relapses in the early relapsing-remitting (ERR) group and two in the benign group; four patients in each group were clinically unchanged. There were 31 new lesions in the ERR of which 26 enhanced, and in the benign group there was one new lesion which also enhanced.

3.2.10. β-adrenoceptor densities on PBMCs (Table 3.3. and 3.4.).

The mean value of β-adrenoceptor density in the 6 normal controls followed in parallel to the MS patients was 1332±140 sites/cell and the (K₀) was 120±40 pM. Enhanced expression of β-adrenoceptors (p<0.05; Wilcoxon rank sum test, compared to NC) was observed in patient 1, 5 and 6 from the relapsing-remitting group and in patient 2, 4 and 6 from the benign group of patients and this was correlated with either clinical or MRI disease activity. The probability that patients in clinical relapse would have high densities of β-adrenoceptors was significant
Similarly, the probability that these patients would have new MRI lesions was significant (p=0.002; \( \chi^2 \) test). There was a fluctuation in densities of \( \beta \)-adrenoceptors reflecting disease activity. The affinity of the receptors when high densities were observed was within the normal range.

3.2.11. High affinity IL-2 receptor densities on PBMCs (Table 3.3 and 3.4.).

The mean±SEM values of IL-2R densities for unstimulated PBMCs from the 6 normal controls followed in parallel to MS patients was 50±10 sites/cell and the affinity \((K_0)\) was 4.7±11.5 pM. Within the 10-150 pM range of concentrations the binding was saturable, detecting the population of receptors with high affinity. Scatchard regression analyses of binding are presented in Fig 3.5, for PBMCs of normal controls, MS patients in relapse, and cells stimulated by PHA in the presence and absence of isoproterenol. The affinity of the receptors was similar in all assayed groups. There was a significantly increased density of IL-2Rs (p<0.05 Wilcoxon rank sum test compared to NC) in patients 1, 2, 4, 5 and 6 of the relapsing-remitting group and 2, 4, and 6 from the benign group, which was correlated with either clinical or MRI disease activity. The probability that high expression of IL2-Rs is more likely to occur when the patients had a clinical relapse or new MRI lesions was respectively p=0.002 of the
Fisher's exact test and p=0.002 of the $\chi^2$ test. There was a statistically significant correlation (p<0.001) between enhanced expression of $\beta$-adrenoceptors and IL-2Rs (Fig 3.6.).

In Table 3.5. are summarized our observations of $\beta$-adrenoceptor and IL2-R densities on PBMCs in relation to clinical or MRI disease activity. MRI activity was observed on 15 occasions and clinical activity on 9 occasions. There was equally strong correlation between clinical activity and enhanced expression of $\beta$-adrenoceptors or IL2-Rs. There was a stronger correlation between MRI activity (enhanced and new MRI lesions) and IL2-R expression than with $\beta$-adrenoceptor enhanced expression. In the absence of MRI activity, increased expression of IL2-Rs and $\beta$-adrenoceptors was observed on respectively 5 and 6 occasions and of these cases respectively 4 and 3 belonged to the benign group of MS patients.

3.2.12. IL-2R densities on PBMCs from NC and MS patients after stimulation with PHA in the presence or absence of isoproterenol.

IL-2R expression on PBMCs from MS patients and NC after stimulation with PHA for 48 hours in the presence and absence of isoproterenol are presented in Fig 3.7. Isoproterenol suppresses IL-2R expression in a similar
manner in PHA-stimulated cells in NC, MS patients in relapse and MS patients in remission. PHA-stimulated cells from MS patients in relapse and NC demonstrate similar densities of IL-2Rs but cells from MS patients in remission demonstrate lower densities of IL-2Rs ($p<0.05$) (Fig 3.7.).

3.2.13. PHA-induced lymphocytic proliferation in the presence and absence of isoproterenol in NC and MS patients.

In Fig 3.8. are presented the proliferation rates of PBMCs from NC, MS patients in remission and MS patients in relapse following stimulation with PHA in the presence or absence of isoproterenol. The rate of proliferation following either treatment was similar in all studied groups. In the presence of $10^{-5}$ M Isoproterenol a significant suppression of proliferation relative to PHA-stimulated PBMCs was observed; 40% in NC and MS patients in remission and 55% in MS patients in relapse.

3.2.14. IL-2R expression on PBMCs from MS patients during relapse before and after stimulation with isoproterenol.

The expression of IL-2Rs on PBMCs from 3 MS patients in clinical relapse was assessed before and after treatment with isoproterenol for 24 hours. In the presence of isoproterenol the high expression of IL-2R returned to the normal range (Fig 3.9.)
Scatchard plot regression analysis of bound versus free (B/F) ligand. The lines from right to left represent linear regression analysis of binding in PBMCs from NC stimulated with PHA after 48 hours with high density of IL-2 receptors (1340 sites/cell, Kd = 4.9 pM), PBMCs stimulated with PHA in the presence of $10^{-4}$ isoproterenol (IL-2R density 789 sites/cell, Kd = 3.9 pM), PBMCs from MS patients in relapse (IL-2R density 350 sites/cell, Kd = 3.8 pM) and PBMCs from NC (IL-2R density 55 sites/cell, Kd = 3.7 pM). The binding in all assays was saturable and the regression analysis demonstrates that the affinity of the receptors was similar (expressed as Kd).
Table 3.3. β-Adrenoceptor and IL-2 receptor densities on PBMCs, clinical status, disability score and MRI findings from six relapsing remitting multiple sclerosis patients in a follow-up study of six months.

<table>
<thead>
<tr>
<th>Months of follow up</th>
<th>1st</th>
<th>2nd</th>
<th>3rd</th>
<th>4th</th>
<th>5th</th>
<th>6th</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-adrenoceptors</td>
<td>2751</td>
<td>2882</td>
<td>1048</td>
<td>3192</td>
<td>2358</td>
<td>1703</td>
</tr>
<tr>
<td>sites/cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>patient 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>age: 27</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sex: female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2 receptors</td>
<td>350</td>
<td>250</td>
<td>100</td>
<td>250</td>
<td>293</td>
<td>238</td>
</tr>
<tr>
<td>sites/cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>clinical status</td>
<td>RL</td>
<td>RL</td>
<td>RM</td>
<td>RL</td>
<td>RL</td>
<td>RM</td>
</tr>
<tr>
<td>Kurtzke score</td>
<td>3.5</td>
<td>4</td>
<td>4</td>
<td>6</td>
<td>5.5</td>
<td>5.5</td>
</tr>
<tr>
<td>MRI lesions</td>
<td>1E</td>
<td>3E</td>
<td>3E</td>
<td>1E</td>
<td>2E,</td>
<td>1E</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-adrenoceptors</td>
<td>1742</td>
<td>1040</td>
<td>1336</td>
<td>1323</td>
<td>1350</td>
<td>1170</td>
</tr>
<tr>
<td>sites/cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>patient 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>age: 41</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sex: female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2 receptors</td>
<td>50</td>
<td>60</td>
<td>150</td>
<td>58</td>
<td>60</td>
<td>47</td>
</tr>
<tr>
<td>sites/cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>clinical status</td>
<td>RM</td>
<td>RM</td>
<td>RM</td>
<td>RM</td>
<td>RM</td>
<td>RM</td>
</tr>
<tr>
<td>Kurtzke score</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>MRI lesions</td>
<td></td>
<td></td>
<td>1NE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-adrenoceptors</td>
<td>1113</td>
<td>1244</td>
<td>1572</td>
<td>1336</td>
<td>1296</td>
<td>1283</td>
</tr>
<tr>
<td>sites/cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>patient 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>age: 26</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sex: female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2 receptors</td>
<td>52</td>
<td>47</td>
<td>60</td>
<td>51</td>
<td>49</td>
<td>48</td>
</tr>
<tr>
<td>sites/cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>clinical status</td>
<td>RM</td>
<td>RM</td>
<td>RM</td>
<td>RM</td>
<td>RM</td>
<td>RM</td>
</tr>
<tr>
<td>Kurtzke score</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>MRI lesions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient</td>
<td>Age</td>
<td>Sex</td>
<td>Beta-adrenoceptors sites/cell</td>
<td>IL-2 Receptors sites/cell</td>
<td>Clinical Status</td>
<td>Kurtzke Score</td>
</tr>
<tr>
<td>---------</td>
<td>-----</td>
<td>-----</td>
<td>-----------------------------</td>
<td>--------------------------</td>
<td>----------------</td>
<td>--------------</td>
</tr>
<tr>
<td>4</td>
<td>45</td>
<td>Female</td>
<td>1224, 1637, 1336, 1290, 1244, 1570</td>
<td>70, 44, 45, 60, 47, 273</td>
<td>RM, RM, RM, RM, RM, RM</td>
<td>2.5, 2.5, 2.5, 2.5, 2.5, 2.5</td>
</tr>
<tr>
<td>5</td>
<td>36</td>
<td>Female</td>
<td>1519, 3235, 2255, 3807, 3206, 1886</td>
<td>49, 450, 250, 360, 390, 75</td>
<td>RM, RL, RL, RL, RM, RM</td>
<td>3.5, 3.5, 3.5, 4, 4, 4</td>
</tr>
<tr>
<td>6</td>
<td>25</td>
<td>Female</td>
<td>2105, 917, 996, 1050, 1167, 1048</td>
<td>370, 40, 52, 55, 53, 52</td>
<td>RM, RM, RM, RM, RM, RM</td>
<td>1.5, 1.5, 1.5, 1.5, 1.5, 1.5</td>
</tr>
</tbody>
</table>

E = enhanced, NE = nonenhanced MRI lesions, RL = relapse, RM = remission. Bold face numbers indicate a significant increase (Wilcoxon rank sum test \( p < 0.05 \)). The probability of coexistence of enhanced expression of beta-adrenoceptors, IL-2-Rs and MRI and clinical activity was significant (\( \chi^2 \) test and Fisher's exact test).
Table 3.4. \( \beta \)-Adrenoceptor and IL-2 receptor densities on PBMC, clinical status, disability score and MRI findings from six benign multiple sclerosis patients in a follow-up study of six months.

<table>
<thead>
<tr>
<th>Months of follow up</th>
<th>1st</th>
<th>2nd</th>
<th>3rd</th>
<th>4th</th>
<th>5th</th>
<th>6th</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \beta )-adrenoceptors</td>
<td>patient 1</td>
<td>1336</td>
<td>1558</td>
<td>1624</td>
<td>1252</td>
<td>1244</td>
</tr>
<tr>
<td>sites/cell</td>
<td>age: 49</td>
<td>IL-2 receptors</td>
<td>40</td>
<td>45</td>
<td>50</td>
<td>55</td>
</tr>
<tr>
<td>sex: female</td>
<td>clinical status</td>
<td>RM</td>
<td>RM</td>
<td>RM</td>
<td>RM</td>
<td>RM</td>
</tr>
<tr>
<td>Kurtzke score</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>MRI lesions</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>( \beta )-adrenoceptors</td>
<td>patient 2</td>
<td>1034</td>
<td>1240</td>
<td>2100</td>
<td>2635</td>
<td>1360</td>
</tr>
<tr>
<td>sites/cell</td>
<td>age: 44</td>
<td>IL-2 receptors</td>
<td>55</td>
<td>40</td>
<td>56</td>
<td>150</td>
</tr>
<tr>
<td>sex: male</td>
<td>clinical status</td>
<td>RM</td>
<td>RM</td>
<td>RM</td>
<td>RL</td>
<td>RM</td>
</tr>
<tr>
<td>Kurtzke score</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>MRI lesions</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>( \beta )-adrenoceptors</td>
<td>patient 3</td>
<td>1890</td>
<td>1572</td>
<td>1625</td>
<td>917</td>
<td>1296</td>
</tr>
<tr>
<td>sites/cell</td>
<td>age: 36</td>
<td>IL-2 receptor</td>
<td>200</td>
<td>50</td>
<td>48</td>
<td>41</td>
</tr>
<tr>
<td>sex: male</td>
<td>clinical status</td>
<td>RM</td>
<td>RM</td>
<td>RM</td>
<td>RM</td>
<td>RM</td>
</tr>
<tr>
<td>Kurtzke score</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>MRI lesions</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Patient</td>
<td>$\beta$-adrenoceptors (sites/cell)</td>
<td>IL-2 Receptors (sites/cell)</td>
<td>Age</td>
<td>Sex</td>
<td>Clinical Status</td>
<td>Kurtzke Score</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------</td>
<td>-----------------------------</td>
<td>-----</td>
<td>-----</td>
<td>----------------</td>
<td>--------------</td>
</tr>
<tr>
<td>4</td>
<td>1113 1446 1336 2690 1294 1165</td>
<td>46 59 150 120 47 48</td>
<td>45</td>
<td>Female</td>
<td>RM RM RM RM RM RM</td>
<td>3 3 3 3 3</td>
</tr>
<tr>
<td>5</td>
<td>1401 1048 1270 1176 1414 1296</td>
<td>49 65 48 44 60 51</td>
<td>54</td>
<td>Female</td>
<td>RM RM RM RM RM RM</td>
<td>2.5 2.5 2.5 2.5 2.5 2.5</td>
</tr>
<tr>
<td>6</td>
<td>1021 1296 996 1250 2120 1348</td>
<td>47 55 52 55 127 55</td>
<td>54</td>
<td>Female</td>
<td>RM RM RM RM RL RM</td>
<td>1.5 1.5 1.5 1.5 1.5 1.5</td>
</tr>
</tbody>
</table>

E=enhanced, NE=norenchanced. MRI lesions RL=relapse, RM=remission. Bold face numbers indicate a significant increase (Wilcoxon rank sum test p<0.05).
TABLE 3.5. Observations in which PBMC β-adrenoceptor (β-ADR) densities are above 2000 sites/cell and IL2-R densities are above 100 sites/cell (These densities are higher than the mean density plus two standard deviations of the normal subjects).

<table>
<thead>
<tr>
<th></th>
<th>MRIenhanced and new lesions</th>
<th>No MRIactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TotalObservations</strong></td>
<td>n=15</td>
<td>n=57</td>
</tr>
<tr>
<td>IL2-Rs86%</td>
<td></td>
<td>IL2-Rs9%</td>
</tr>
<tr>
<td>β-ADRs53%</td>
<td></td>
<td>β-ADRs11%</td>
</tr>
<tr>
<td><strong>Clinical Relapse</strong></td>
<td>n=9</td>
<td>n=7</td>
</tr>
<tr>
<td>IL2-Rs100%</td>
<td>IL2-Rs100%</td>
<td>IL2-Rs100%</td>
</tr>
<tr>
<td>β-ADRs100%</td>
<td>β-ADRs100%</td>
<td>β-ADRs100%</td>
</tr>
<tr>
<td><strong>Clinical Remission</strong></td>
<td>n=63</td>
<td>n=8</td>
</tr>
<tr>
<td>IL2-Rs14%</td>
<td>IL2-Rs75%</td>
<td>IL2-Rs5%</td>
</tr>
<tr>
<td>β-ADRs8%</td>
<td>β-ADRs12%</td>
<td>β-ADRs9%</td>
</tr>
</tbody>
</table>
Correlation between β-adrenoceptor and IL-2R expression on PBMCs in MS. Correlation between β-adrenoceptor and IL-2R densities in all studied MS patients. The Y axis represents the IL-2R densities and the X axis the β-adrenoceptor densities. There is a significant correlation with $r=0.81$ and $p<0.001$, between the two measurements in each patient at each time.
Fig 3.7. IL-2Rs on PBMCs from MS patients in the presence of PHA and Isoproterenol.

PBMCs from 6 relapsing-remitting MS patients, 6 benign MS patients and 6 NC were cultured at each monthly assessment for 48 hours in the presence of PHA (filled bar) and PHA and isoproterenol $10^{-4}$M (crossed bar). In NC IL-2R density was higher in the presence of PHA (1400±30 sites/cell) compared to that in the presence of PHA+isoproterenol (590±20 sites/cell). +p<0.01 t-student paired test. In MS patients in relapse IL-2R density was higher in the presence of PHA (1387±69 sites/cell) compared to that in the presence of PHA+Isoproterenol (486±41 sites/cell). *p<0.01 t-student paired test. In MS patients in remission IL-2R densities after PHA stimulation increased to 782±28 sites/cell which is significantly lower to the increase observed in NC and MS patients in relapse. °p<0.02 compared to stimulated levels of NC and MS patients in relapse (Wilcoxon rank sum test), and p<0.02 compared to the isoproterenol-stimulated levels. The graphic presentation is in means±s.e.m, despite the use of non-parametric statistical analysis test.
Fig 3.8. Proliferation Rate of PBMCs from MS patients stimulated with PHA and Isoproterenol.

$[^{3}H]$ Thymidine incorporation after stimulation with PHA (1μg/ml cell concentration $1 \times 10^6$ cells/ml) (filled bar) and PHA+isoproterenol $10^{-4}$M (crossed bar) in NC, MS patients in remission and MS patients in relapse. Proliferation is similar in all groups. There is a significant suppression of proliferation in the presence of isoproterenol being 60% of the total in NC and MS patients in remission and 45% of the total in MS patients in relapse. *p<0.01 t-student paired test.
Fig 3.9. IL-2Rs in relapsed MS patients after stimulation with Isoproterenol.

PBMCs from three MS patients in clinical relapse were cultured in the presence or absence of isoproterenol $10^{-4}$M for 24 hours. In the absence of isoproterenol a high density of IL-2Rs was observed 213±15 sites/cell, while in the presence of isoproterenol the density of IL-2Rs returned to the normal range values 58±20 sites/cell.*p<0.01 t-student paired test.
3.3. Circulating cytokines in MS patients.

Several lines of evidence have implicated cytokine involvement in the inflammatory demyelinating process in MS. Changes in circulating cytokines have been reported to be associated with clinical activity in MS but results from CSF and serum determinations in different laboratories have produced conflicting data. In some reports, IL-1 and TNFα have been identified as markers of relapse (Frei et al., 1991; Gallo et al., 1991), while in others no correlation has been found between CSF and serum cytokine levels and disease activity (Hauser et al., 1990; Peter et al., 1991; Maimone et al., 1991; Franiciotta et al., 1989; Tsukada et al., 1991; Sharief et al., 1991).

Since both IL-1 and IL-2 appear to be involved in the up-regulation of β-adrenoceptors on PBMCs in vitro, further information in relation to the association between the inflammatory process and β-adrenoceptors in MS could be provided by the assessment of levels of circulating cytokines in MS patients.

3.3.1. Methods.

3.3.2. Levels of TNFα, IL-1α, IL-1β, IL-2 and IL-6.

Serum samples were collected and frozen in 1ml aliquots in sterile vials at -70°C, until required.
Cytokine levels in the samples were determined by enzyme linked immunoassay using R+D systems kits, supplied by British Biotechnology (Oxford, UK). Assays were performed as given in the manufacturers' instructions. The cytokines assayed were as follows and the level of sensitivity for each assay are given in parentheses: TNFα (7.5pg/ml), IL1α (7pg/ml), IL1β (0.3pg/ml), IL2 (10pg/ml) and IL6 (0.35pg/ml). Standard curves for each cytokine were included in all assays. For non-detectable levels in assayed samples the value of zero (0) was given.

3.3.3. Cytokine serum levels.

Serial cytokine measurements in 2 NC, patients 1, 5 and 6 from the relapsing-remitting MS group (Table 3.3.) and patients 2 and 6 from the benign MS group (Table 3.4.) were performed [12 samples of serial measurements in 2 NC, 9 samples of serial measurements in relapse and 21 samples of serial measurements in remission of MS]. There was a significant increase (p<0.05; Wilcoxon rank sum test) in serum levels of TNFα (120±50 pg/ml) and IL1β (20±7.5 pg/ml) in MS patients during relapse compared to NC (TNFα:1.5±0.81 pg/ml and IL1β:2.2±0.53) and of IL1β (13.7±4 pg/ml) in MS patients during remission compared to NC (Fig 3.10.).
Fig 3.10. Cytokine Plasma Levels in MS patients.

Logarithmic expression of mean±sem serum levels of TNFα, IL-1α, IL-1β, IL-2 and IL-6 in 2 NC and 5 MS patients assessed monthly. In NC values were 1.43±0.81, 0.03±0.08, 2.21±0.53, 0.46±0.29 and 1.15±0.24 pg/ml, in MS patients in remission were 2.69±0.80, 0.76±0.46, 13.72±4.06, 2.91±1.31 and 0.92±0.20 pg/ml and in MS patients in relapse were 120±49.89, 0.65±0.23, 19.94±7.55, 3.81±1.69 and 15.03±10.75 pg/ml for TNFα, IL-1α, IL-1β, IL-2 and IL-6 respectively. *p<0.05 (Wilcoxon rank sum test) compared to the NC levels. The graphic presentation is in means±s.e.m., despite the use of non-parametric statistical analysis test.
3.3.4. Cytokines after stimulation of whole blood with PHA.

Serial cytokine measurements were also performed on serum from the above-mentioned NC and MS patients after stimulation of whole blood with PHA at a concentration of 1μg/ml for 3 hours. Significantly increased production of IL-1β in response to stimulation was observed in MS patients during relapse. No other difference in production of cytokine after stimulation was observed in MS compared to NC (Fig 3.11.).

3.4. Discussion.

This study has confirmed that β-adrenoceptor density on PBMCs is increased during a relapse of disease in relapsing-remitting MS patients. In a serial investigation of twelve patients over a six month period we have also demonstrated fluctuations in IL-2R expression on PBMCs which mirror disease activity. The significant correlation between PBMC β-adrenoceptor density and IL-2R expression (Fig 3.6.) and the association with indicators of disease activity suggest that both are sensitive markers of a relapse.

IL-2R expression in MS has been investigated by estimating soluble IL-2Rs or counting total IL-2R (Tac) positive cells. As it has been postulated that the T-cell
Fig 3.11. Production of Cytokines after stimulation with PHA in NC and MS.

Logarithmic expression of mean±sem levels of produced TNFa, IL-1α, IL-1β, IL-2 and IL-6 in 2 NC and 5 MS patients assessed monthly. In NC values were 401±57, 2.33±1, 90±17, 1.70±0.82 and 34±8.3 pg/ml, in MS patients in remission were 298±46, 0.46±0.22, 176±39, 1.52±0.3 and 71±9.3 pg/ml and in MS patients in relapse were 351±78, 2.12±0.81, 274±39, 1.78±0.82 and 97±28 pg/ml for TNFa, IL-1α, IL-1β, IL-2 and IL-6 respectively. *p<0.05 (Wilcoxon rank sum test) compared to the NC levels. The graphic presentation is in mean±s.e.m., despite the use of non-parametric statistical analysis test.
response is mediated via the high affinity receptor (Robb et al., 1984), the radioligand binding assay used in this study served two purposes: first the assessment of the high affinity receptors on intact cells and secondly the investigation of in vitro interactions between the β-adrenoceptor and IL-2R expression. Since Gadolinium enhanced MRI lesions indicate an early phase of blood-brain barrier breakdown (Kermode et al., 1990) and frequently appear without clinical signs, correlation with immune parameters such as expression of IL-2Rs on PBMCs, could further clarify aspects of the natural history of MS and validate the use of MRI in monitoring disease activity.

Analysis of our findings (Table 3.5.), confirms earlier observations (Miller et al., 1988) of disease activity without clinical signs, as clinical relapses were recorded on 9 occasions, while MRI activity was present on 15 occasions. The enhanced expression of β-adrenoceptors correlated more strongly with clinical activity, while that of IL-2Rs was more closely associated with MRI activity. Only on 2 occasions was there no correlation with IL-2R expression (patient 5 and 6; Table 3.3.), and in both cases this was associated with disease activity in preceding or following observations. It was of interest that the majority of the observations in which high β-adrenoceptors and IL-2Rs did not correlate with disease activity belonged to the benign group of patients. From
these limited observations, it would appear that there is greater immunological than clinical or MRI evidence of disease activity in benign MS patients. The more frequent recording of immunological and MRI evidence of disease activity, and the better correlation between MRI activity and IL2-R expression indicate that MRI findings mirror more accurately disease activity in MS.

IL-2R expression in vitro appears to be more tightly controlled than that of β-adrenoceptors under conditions of mitogenic stimulation, although both are associated with lymphocyte activation. To further clarify the role of β-adrenoceptors in immunoactivation in MS, we evaluated IL-2R expression following stimulation of the cAMP second messenger system (Feldman et al., 1987; Anastasiou et al., 1992). Suppression of IL-2R expression by isoproterenol (a β-receptor agonist) was observed on proliferating lymphocytes from NC and MS patients in relapse and remission. However, IL-2R expression after mitogenic stimulation appeared to be lower in remitting MS patients compared to NC and MS patients in relapse possibly indicating a relative immunosuppression during disease remission (Fig 3.7.). Similarly, the proliferation rate of PBMCs was suppressed after stimulation with isoproterenol by 40% in NC and MS patients in remission and by 55% in MS patients in relapse. The higher suppression in MS patients during relapse may mirror the enhanced function of PBMC β-adrenoceptors in those patients (Fig 3.7.).
It has been suggested that the existence of a high density of $\beta$-adrenoceptors on PBMCs in MS could represent a significant recovery mechanism (Zoukos et al., 1992a). This was further supported by in vitro studies in which isoproterenol was found to be effective in reducing the IL-2R density in PBMCs from MS patients in relapse, to within the normal range (Fig 3.9.). However, the precise timing of these changes during the disease process is of importance if it is to be exploited therapeutically by $\beta$-adrenergic agonists or antagonists.

Immune activation results in release of various cytokines which can propagate the immunoreactive and inflammatory process. As in vitro studies have demonstrated that leukocyte $\beta$-adrenoceptor density is increased in the presence of interleukins -1 and -2 as well as cortisol, we have assessed the levels of five potentially proinflammatory cytokines. The increase in plasma TNF$\alpha$ in MS patients in relapse confirms other observations (Beck et al., 1988; Tsukada et al., 1991), although there is a lack of consensus about the value of plasma cytokines as markers of disease activity in MS. The increased IL-1$\beta$ serum levels, observed in both relapsing and remitting phases of the disease and after stimulation of whole blood in relapsed MS patients, indicate a persisting monocyte activation (Beuscher et al., 1992). However, the small number of serial plasma level cytokine measurements gives only some indication of pattern
changes, and a larger serial study will be required in order to confirm those changes.

In conclusion this study clearly demonstrates that alterations in expression of lymphocyte β-adrenoceptors and IL-2Rs correlate with disease activity in MS. It appears that β-adrenoceptor enhanced expression closely follows activation of the lymphocytes. The inhibition of IL-2R expression after β-adrenoceptor stimulation in vitro suggests that increased number of β-adrenoceptors could constitute a recovery mechanism in MS. The alterations in peripheral blood in association with central nervous system (CNS) pathology suggests a systemic immunoreactive process in MS, rather than one confined to the CNS. The observed correlations between MRI findings and IL2-R changes on PBMCs indicate that both are useful markers of disease activity.
4.1 Autonomic Nervous System

The autonomic nervous system innervates every organ in the body, creating as Galen suggested, 'sympathy' between the various parts of the body. It has as complex a neural organization in the brain, spinal cord, and periphery as the somatic nervous system, but remains largely involuntary or autonomic. Although initial attention was given to the distinction between cholinergic and adrenergic transmission in the autonomic nervous system, nosological entities diverted our attention to the two anatomically discrete parts of it, the central and the peripheral.

4.1.1. Peripheral autonomic function.

The peripheral autonomic nervous system, an afferent system, is made up of neurons that lie outside the central nervous system and that are concerned with visceral innervation. Both sympathetic and parasympathetic systems have preganglionic neurones in the brain and spinal cord arranged as shown in Fig 4.1. The transmitter at all preganglionic terminals is acetylcholine. Noradrenaline is
4.1. **Peripheral autonomic system.**

The sympathetic innervation of the vessels, sweat glands, and piloerector muscles is not shown. Solid lines, pregangionic axons (from Brain 1985).
the principal transmitter for postganglionic sympathetic
nerves, but there are a few areas where there is
cholinergic transmission. Noradrenaline is stored in the
terminals and is released by nerve activity or by
sympathomimetic drugs, which may act partly indirectly on
the ganglia or more centrally. The different actions of
noradrenaline and adrenaline are caused by relative
effects on different receptors. α-1 may be either
postsynaptic (α1) or presynaptic (α2, which when stimulated
decrease the release of the transmitter). α2- are also
present at the postsynaptic level and they mediate
vasoconstriction when stimulated with agonists. β-
receptors mediate vasodilatation, especially in muscles,
increase the rate and the force of the heart and cause
bronchial relaxation. These receptors are further divided
into β1 and β2 receptors. The β2-adrenoceptors are
responsible for most of the peripheral effects of the β-
adrenergic stimulation. The autonomic nervous fibres of
the lymphoid glands may exert their influence upon PBMCs
through β2-adrenoceptors. Though descriptions of autonomic
sensitivity phenomena were first made more than a century
ago, the research was summarized by Cannon and Rosenblueth
in 1949 under the title, The supersensitivity of
denervated structures: a law of denervation. Attention
since then has concentrated on the 'up' and 'down'
regulation of receptor function depending on the
availability of the transmitter.
The cells of the autonomic nervous system tend to act in conjunction and this is achieved mainly by specialized intercellular junctions at the ganglion cells. The autonomic ganglia also contain small intensely fluorescent cells (‘SIF’ cells) which contain many peptides, thought to act as modulators and transmitters at synaptic sites. Substance P, VIP, encephalins, and somatostatin have all been identified in autonomic ganglia although their precise role in control of nerve transmission is not yet known.

4.1.2. Central control of the autonomic nervous system.

The hypothalamus can be considered the ‘highest’ level of integration of autonomic function. It remains under the influence of the cortex and the group of structures known as the ‘limbic system’, which includes the olfactory areas, the hippocampus and amygdaloid complex, the cingulate cortex, and the septal area. These regions of the brain regulate the hypothalamus and are critical for emotional and affective expression. The hypothalamus is also concerned with maintaining homeostasis against a changing environment. The autonomic nervous system and many metabolic functions are under the control of the limbic system by means of nerve centres, many of which are situated in the hypothalamus, lying ventrally to the thalamus and constituting the floor of the third ventricle. The hypothalamus contains a large
number of scattered ganglion cells, which have been differentiated into a number of nuclei (Appenzeller 1990). The projections of the hypothalamus are not yet completely known. The hypothalamus controls the autonomic nervous system in two ways, by means of the pituitary and hence other endocrine glands and by direct descending nervous pathways. Despite these descending pathways, some regions of the brain stem are to some extent autonomous and function in animals after pontine section of the brain stem. These include cardiac and respiratory function and 'centres' for vomiting and micturition. The integration of these changes takes place in the hypothalamus. The main course taken by descending sympathetic fibres from the hypothalamus is uncrossed and by way of the lateral tegmentum of the brain stem and lateral medullary formation. Some fibres end directly on the intermediolateral column cells, while others synapse in the reticular formation.

4.1.3. Diseases of the autonomic nervous system.

Systematic application of physiological techniques of study to patients with autonomic failure started in the 1960s. Research interest in postural hypotension, the usual presenting symptom of autonomic failure, was stimulated by its occurrence after the weightlessness of space travel. Since then there have been striking advances in the investigation and classification of the syndromes
of autonomic failure which were until recently both confused and confusing. Peripheral neuropathies with an autonomic component have long been recognized, particularly in diabetes, alcoholism and amyloid. Sharpey-Schafer and Taylor (1960) showed that the sympathetic vasoconstrictor pathway to the hands was intact in diabetic autonomic neuropathy, and they therefore attributed the absence of circulatory reflexes and the postural hypotension to an afferent lesion.

However, the major interest lies in the chronic or primary neurological disorders in which the autonomic nervous system is selectively involved by both pre- and postganglionic neuronal degeneration (Shy and Drager 1960; Johnson et al. 1966; Bannister et al 1967). A large number of diseases of largely known pathology such as diabetes when they present with clinical signs of autonomic dysfunction could be classified as 'secondary' autonomic failure. Other patients, without certainly known pathology in common, share certain autonomic symptoms and, from tests in life and observation of similar patients after death, we choose to use the word 'primary' disease. For this group of patients, in whom autonomic failure appears to result from a primary or unexplained selective neuronal degeneration, this may occur in a 'pure' form without other neurological signs, or it may occur in association with two quite different degenerations of the nervous system, multiple system atrophy and Parkinson's
Historically, the first reported cases of autonomic failure were described by Bradbury and Eggleston (1925) as 'idiopathic orthostatic hypotension'. This term is misleading because it stresses only one feature of autonomic failure and ignores the more usually associated neurological disturbances of bladder, sexual function, and sweating, and also because the word 'idiopathic' implies that it is a single disease entity, which is not proven. The term 'pure autonomic failure' (PAF) is now accepted generally for this syndrome. The cases which were originally described by Shy-Drager and appeared with autonomic failure features, Parkinsonism, cerebellar signs and pyramidal signs, are now called Multiple System Atrophy (MSA) or Shy-Drager syndrome. Additionally, autonomic failure may rarely also associated with otherwise apparently typical Parkinson's disease (PD) (Fichefet et al. 1965; Vanderhaegen et al. 1970).

There is a good evidence that virtually all patients with primary autonomic failure as opposed to secondary autonomic failure, studied at post-mortem, have severe loss of intermediolateral column cells, the final common pathway cell for the sympathetic nervous system. It is becoming more probable that the pathological process, whether viral, biochemical, immunological, or of some other kind, that leads to this loss of intermediolateral
column cells differs significantly in PAF (and probably in autonomic failure with PD) from that in autonomic failure with MSA. In PAF there appears to be an additional loss of ganglionic neurons which are relatively intact in MSA. This suggests the existence of a more distal process in PAF than in MSA.

4.1.4. β-adrenoceptor densities on PBMCs in autonomic failure patients.

β-adrenoceptors are found in various cardiac tissues, in most blood vessels, in the bronchi and intestine, in the central nervous system and on lymphocytes. Lymphocytes possess β2-adrenoceptors and as there is evidence for a good correlation between their number and the number of β2-adrenoceptors in heart and lung cells (Brodde et al., 1987; Liggett et al., 1988), their accessibility for in vivo studies is of importance. In patients with pure autonomic failure and with autonomic failure plus multiple system atrophy (MSA), there is a denervation supersensitivity of β-adrenoceptors shown by the greater chronotropic effect on the heart and steeper dose response curve for isoprenaline (Bannister et al., 1981). Patients in studies of adrenoceptor function and autonomic failure tend to be old, but this fact highlights further the effect of the autonomic failure because older people tend to have decreased β-receptor response and probably decreased α-receptor responses. In patients with autonomic
failure and MSA who were supersensitive to intravenous isoproterenol, \( ^3 \)H-dihydroalprenolol (DHA) binding showed an increased number of \( \beta \)-adrenoceptors on circulating lymphocytes (Bannister et al., 1981; Davies 1982; Kilfather et al., 1985).

However, the affinity of the \( \beta \)-adrenoceptors, as measured by equilibrium association constants for DHA binding, was not different in MSA lymphocytes compared with normals (Bannister et al., 1981). In spite of the difficulty of extrapolating from cardiac \( \beta_1 \)-receptors, which mediate the \textit{in vivo} chronotropic isoprenaline response, to \textit{in vitro} lymphocyte \( \beta_2 \)-receptors, the increase in \( \beta \)-receptors observed on lymphocytes from MSA subjects, suggest that increased \( \beta \)-receptor numbers are likely to be important in the \textit{in vivo} denervation supersensitivity to isoprenaline. Patients with pure autonomic failure and autonomic failure with MSA often have lower concentrations of endogenous catecholamines than normal so that the increased number of \( \beta \)-receptors seen in these patients may reflect agonist regulation of adrenoceptor numbers (Davies et al., 1984). \( \beta \)-adrenoceptors appear to mediate their effects via stimulation of the adenylate cyclase enzyme complex to which they are linked. In one study of lymphocytes from patients with MSA, isoprenaline-stimulated cAMP generation was three-to fivefold greater than for normal lymphocytes. (Jennings et al., 1981) but this was not confirmed by Kilfeather et al 1985.
Teleologically, β-adrenoceptor antagonist treatment can be viewed as partial pharmacological autonomic failure for endogenous catecholamines are denied access to tissue β-receptor. Sudden β-antagonist withdrawal has been reported to cause tachyarhythmias, angina, and even myocardial infarction in patients treated chronically with these drugs. This β-blocker withdrawal syndrome may be associated with β-adrenoceptor supersensitivity and some studies have shown increased lymphocyte β-adrenoceptors in patients after β-antagonist withdrawal (Aarons et al., 1980). Similarly, supersensitivity in autonomic failure patients could be attributed to increased β-adrenoceptors, which has been associated with an inverse correlation with the plasma levels of noradrenaline and adrenaline.

4.1.5. Autonomic function and expression of β-adrenoceptors on PBMCs in multiple sclerosis patients.

Autonomic dysfunction affecting sudomotor, urinary bladder, bowel, sexual organs and cardiovascular system has been described in MS. Increases of β-adrenoceptors on PBMCs has also been reported in progressive and relapsing-remitting MS patients. This finding could be attributed to sympathetic dysfunction, inflammation or a combination of the two (Karaszewski et al., 1990; Zoukos et al., 1992a). Any attempt to separate the contribution of autonomic dysfunction in β-adrenoceptor overexpression in MS, would
require the investigation of β-adrenoceptor expression on PBMCs and physiological, haemodynamic and neurohormonal responses in progressive MS patients, autonomic failure patients (AF) and normal subjects.

In this context, a physiological assessment of autonomic function in MS patients and a study of the neurohormonal, cardiovascular and haemodynamic responses to a centrally acting sympatholytic agent clonidine, has been carried out in MS, AF patients and normal subjects.
4.2. Physiological assessment of autonomic function in progressive MS patients.

4.2.1. Methods.

4.2.2. Cardiovascular testing.

The prime concern of the cardiovascular system is tissue perfusion, with blood pressure and blood flow therefore of critical importance. These are influenced by a number of factors, with beat-to-beat control of blood pressure dependent upon the autonomic nervous system and in particular, the sympathetic efferent pathways. A schematic diagram of the main neurological pathways involved in the regulation of the blood pressure is provided in Fig 4.2. There are cortical, limbic, anterior, and posterior hypothalamic and medullary centres, where the input from a range of afferents can be integrated. The major cardiovascular afferents are those from the carotid sinus, the aortic arch, and the cardiopulmonary afferents, although a range of other afferents (from the skeletal muscle, skin, and viscera) also contribute. Normally, from the cerebral centres the output through the vagus and the sympathetic nervous system to the heart and blood vessels is co-ordinated. Lesions resulting in autonomic dysfunction may involve the afferent pathways, the central connections, the efferent pathways, the target organs, or a combination of these.
Fig 4.2. Diagram of cardiovascular control mechanisms.

LC, locus ceruleus; NA, nucleus ambiguous; NTS, nucleus tractus solitarius; IML, intermediolateral column; R, renin.
4.2.3. Postural challenge.

Cardiovascular responses to head-up postural change are of particularly important since postural hypotension is a cardinal feature in autonomic failure. Postural hypotension is arbitrarily defined as a fall of more than 20 mm Hg systolic blood pressure on standing. Brachial blood pressure in this study was measured non-invasively using an automated sphygmomanometer (Sentron). Basal measurements were performed in a temperature controlled room ($24^\circ C$) after 30 min supine rest to allow equilibration and stabilization. For postural change a tilt table ($45^\circ$) was used for a duration of 10 min. Measurements of blood pressure and heart rate were made at 2 and 10 min. There is normally a small-to-moderate rise in heart rate during postural change. In the presence of a substantial fall in blood pressure, a lack of change in heart rate is indicative of a baroreflex abnormality, as in sympathetic and parasympathetic failure.

4.2.4. Valsalva manoeuvre (VM).

The changes in blood pressure and heart rate during the Valsalva manoeuvre, when intrathoracic pressure is raised ideally to 40 mm Hg, provide a further assessment of the baroreflex pathways. To perform this the subject blows with an open glottis into a disposable syringe connected to the mercury column of a sphygmomanometer and
maintains a forced expiratory pressure of up to 40 mm Hg for 15 sec. With the rise in intrathoracic pressure the venous return falls along with blood pressure (Fig 4.3.). On releasing intrathoracic pressure there is a blood pressure overshoot because of persistence of sympathetic activity. Baroreflex activation results in a secondary fall in heart rate to below basal levels. The Valsalva ratio was calculated by dividing the shortest R-R ECG interval during raised intrathoracic pressure by the longest R-R ECG interval after release.

4.2.5. Pressor stimuli.

These raise blood pressure by stimulating sympathetic efferent pathways in a variety of ways, such as isometric exercise or cutaneous cold through activation of peripheral receptors, although there is often an important cerebral component. Others, such as mental arithmetic, are dependent predominantly on cerebral stimulation.

Isometric exercise was performed by using a partially inflated sphygmomanometer cuff, and sustaining handgrip for 2 min, usually at a third of the maximum voluntary contraction pressure. The cold pressor test consisted of immersing the hand for up to 2 min in ice slush, just below 4° C. Cortical arousal was performed by mental arithmetic test (substraction of 7 out of 100). These
Changes in intraarterial blood pressure and heart rate before, during, and after the Valsalva manoeuvre, when intrathoracic pressure was raised to 40 mm Hg in a normal subject (upper trace) and in a patient (lower trace). In the normal subject release of intrathoracic pressure was accompanied by an increase in blood pressure and a reduction in heart rate below basal levels. In the patient there was a gradual increase in blood pressure implying impairment of sympathetic vasoconstrictor pathways. The heart-rate scale varies in the two subjects. (provided by Prof. C.J. Mathias, Autonomic Unit, The National Hospital for Neurology and Neurosurgery, London).
stimuli normally elevate blood pressure and heart rate (Fig 4.4.).

4.2.6. Heart rate responses to respiratory change.

Changes in respiration result in rapid responses in the cardiac vagi and variations in heart rate often provide a good guide to their activity. Normally, with inspiration there is a rise and with expiration a fall in heart rate, this being the basis of sinus arrhythmia (Fig 4.5.). Hyperventilation is probably a stronger stimulus to vagal withdrawal and causes a rise in heart rate. It may also lower blood pressure; the precise mechanisms of this are unclear. In this study deep breathing (DB) at a rate of six cycles per minute during continues ECG monitoring was analysed.

4.2.7. Noninvasive quantification of superior mesenteric artery (SMA) blood flow during sympathoneural activation.

In man the splanchnic organs receive about 25% of the cardiac output at rest and contain between 20-30% of the total blood volume (Rowell, 1975; Grayson et al., 1965). The splanchnic circulation has been described as the 'blood giver of the circulation' and could play a major role in overall circulatory regulation (Katz et al., 1939; Rowell et al., 1984).
The SMA supplies the whole small intestine and the anatomy of this vessel lends itself to examination by noninvasive Doppler ultrasound method. Also the SMA is almost completely innervated by sympathetic vasoconstrictor splanchnic nerves, with a small contribution from the lumbar sympathetic (Rowell, 1975). Measurements therefore of SMA blood flow (SMABF) in basal state and after different challenging tests could provide information for sympathetic responses in the vascular bed. SMABF was measured using a pulsed Doppler method with a real time, 2-D ultrasonic scanner (Acuson 128 computed sonographic system, 3.5 MHz sector transducer). An upper abdominal longitudinal scan was used. A real time imaging system provided satisfactory visualization of the SMA and enabled placement of Doppler sample volume in the centre of its stream so that Doppler shift signals could be obtained (Fig 4.6.). The sample volume was taken from the first part of the SMA, where its origin and the signals could be readily distinguished from the aorta and neighbouring vessels, particularly the coeliac artery. Signals and images were recorded on videotape and analysed using a built-in spectrum analyzer and computer. Time average velocity (TAV) was calculated from the real time amplitude weighted frequencies in the sample volume with angle correction and using the Doppler formula. Volume blood flow in SMA was then calculated using the TAV and cross-sectioned area at the point of TVA measurement. The diameter of the SMA (mean of three measurements for each
test) and the angle of insonation was calculated using real time imaging. Sequential measurements of the diameter of SMA were taken in supine position and during tilt. Blood flow was then calculated using the formula:

$$\text{SMABF} = \text{TAV} \times \pi \times r \times 60 \text{ ml/min}$$

where $r =$ radius in min.

### 4.2.8. Cardiac index (CI).

Cardiac index was used as a measure of the cardiac output and was calculated by multiplying stroke distance and heart rate. Stroke distance was derived from the integral of the peak velocity profile of ascending aortic blood flow measured by a continuous wave Doppler ultrasound technique (Exerdop; Quinton Instrument Company). This technique has been validated as a measure of cardiac output (Mehta et al., 1985).

### 4.2.9. Forearm muscle blood flow (FBF).

FBF was measured using a mercury-in-silastic strain gauge and standard plethysmographic techniques with recording on a two channel recorder (Ormed). The arm was kept horizontal and at the level of the heart during each measurement and six readings were obtained during each test. Vascular resistance was calculated by dividing the mean arterial pressure by flow.
4.2.10. Digital Skin Blood Flow (DSBF).

Digital skin blood flow was calculated by a laser Doppler flowmetry (Periflux PF 2B). The laser light is delivered to the skin via flexible graded-index fiberoptic light guides. A portion of the incident light strikes nonmoving structures and is reflected with no shift in frequency. The portion striking moving red blood cells is reflected with a shift in frequency (Doppler broadening). The reflected light is guided from the tissue surface through a second fiberoptic light guide, mixed (heterodyned), and analyzed in real time by an analog processor that provides a continuous output of the instantaneous mean Doppler frequency in the photocurrent identified by a square-law detector. The mean Doppler frequency has been predicted by theory to be linearly related to blood flow (Bonner et al., 1981)

4.2.11. Subjects.

Ten patients with secondary progressive MS (mean age: 42±11 years, range 20-45, 4 male, and 6 female) and 10 age and sex matched subjects (controls) (mean age:42±12 years, range 19-55 year) took part in this study. All MS patients were characterized as secondary progressive (Thompson et al., 1991), as they presented with a relapsing-remitting form of the disease but during the year preceding the investigation they entered the progressive phase of the
Fig 4.4. Blood Pressure and Heart rate responses to cutaneous cold.

Blood pressure (BP) and heart rate (HR) responses to cutaneous cold (hand up to wrist in ice slush) (a) in a normal subject and (b) in a patient with autonomic failure. The time scale is similar in (a) and (b). In the patient there is no rise in BP. Non invasive recordings were made with the Finapres. (provided by Prof. C.J. Mathias, Autonomic Unit, The National Hospital for Neurology and Neurosurgery, London).
Fig 4.5. Deep Breathing, Heart Rate and Blood Pressure.

The effect of deep breathing on heart rate and blood pressure in (a) a normal subject and (b) a patient with autonomic failure. There is no sinus arrhythmia in the patient, despite a fall in blood pressure. Respiratory changes are indicated in the middle panel. (Provided by Prof. C.J. Mathias, Autonomic Unit, The National Hospital for Neurology and Neurosurgery, London).
(a) Real time two dimensional colour doppler image showing the superior mesenteric artery (SMA) and coeliac artery arising from the aorta in longitudinal section. The red colour signifies blood flowing towards the transducer. (b) Real time two dimensional colour doppler image showing the SMA with a cursor placed within its lumen so as to obtain the frequency shift signal (shown below). (provided by Dr T. Thomaides, Pickering Unit, St. Mary's Hospital School of Medicine, London).
disease losing at least one point in the Expanded Kurtzke Disability Status Scale (EDSS) (Kurtzke, 1983). Multiple sclerosis was diagnosed on the basis of standard criteria (Poser et al., 1983) and the overall disability score did not exceed seven (7) in the EDSS. T2-weighted magnetic resonance imaging (MRI) of the brain was performed in 9 of the MS patients (Picker, International, Wembley, Middlesex U.K. 0.5 tesla, spin echo (SE) 2000/60, 5-mm continuous slices, 256X256 image matrix). No MS patient was on immunosuppressive or steroid treatment during the last 6 months. None had postural hypotension and there was no evidence of autonomic dysfunction in these patients apart from bladder symptoms in 8 patients. All patients were shown to have up-regulated β-adrenoceptor densities on PBMCs. Their age, duration of the illness, neurological deficits and disability score are presented in Table 4.1. All subjects were studied after an overnight fast in a temperature controlled room (24°C). All measurements were made after 30 min supine rest to allow equilibration and stabilization. Subjects, non-randomly, underwent stimuli which included mental arithmetic (MA), cutaneous cold (CC), isometric exercise (ISE), Valsalva manoeuvre (VM), deep breathing (DB) and head-up tilt. A ten minute period of equilibration was allowed between each stimulus and measurements were made before and during each stimulus. All studies were carried out at the Autonomic Unit of the National Hospital for Neurology and Neurosurgery by Dr Y. Zoukos and Dr T. Thomaides. MS patients were selected and
assessed clinically by Dr Y. Zoukos. For the studies Ethical Approval from the Ethics Committee of the National Hospital for Neurology and Neurosurgery, had been obtained.

Table 4.1. Multiple sclerosis patients who were tested. Age, sex, duration of the disease, bladder symptoms and disability score (EDSS).

<table>
<thead>
<tr>
<th>N</th>
<th>Age (years)</th>
<th>Duration (years)</th>
<th>Bladder symptoms</th>
<th>EDSS score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>44♂</td>
<td>15</td>
<td>+</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>39♂</td>
<td>7</td>
<td>+</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>42♀</td>
<td>15</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>23♂</td>
<td>3</td>
<td>+</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>61♀</td>
<td>13</td>
<td>+</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>43♀</td>
<td>11</td>
<td>+</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>35♀</td>
<td>10</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>8</td>
<td>42♀</td>
<td>13</td>
<td>+</td>
<td>7</td>
</tr>
<tr>
<td>9</td>
<td>33♀</td>
<td>6</td>
<td>+</td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td>49♂</td>
<td>12</td>
<td>+</td>
<td>7</td>
</tr>
</tbody>
</table>

9 out of 10 patients had an MRI brain scan. All of them had periventricular and diffuse white matter lesions.

4.2.12. Statistical analysis.
Statistical analysis was carried out using a standard version Minitab data software (Minitab, Inc 1989). Blood pressure (BP), heart rate (HR), Blood Flow (SMBF, FBF) and cardiac index (CI) values before and during each stimulus were compared by paired t-test and p<0.05 was considered significant. Changes in BP, SMBF, and SMA vascular resistance (SMAVR) between controls and MS patients and mean baseline values preceding each test, were compared by analysis of variance (ANOVA). Data are presented as means±s.e.m.

4.2.13. Results.

Changes in blood pressure (BP) and heart rate (HR) in controls and MS patients before and during stimuli are shown in Table 4.2. In controls, the mean arterial blood pressure (MABP) rose during mental arithmetic (MA), cutaneous cold (CC), and isometric exercise (ISE) and it was maintained during tilt at 2 and 10 minutes. In MS patients as a group, MABP was unchanged during MA, CC and ISE and was maintained during tilt (Table 4.2.). When individually considered, there were impaired pressor responses to MA and ISE in six and to CC in four patients Fig 4.7a. Changes in HR were significant only in controls during ISE (Table 4.2.). In controls, CI rose during ISE and fell during tilt at 2 and 10 minute (Table 4.2). In patients with MS, the CI remained unchanged during the pressor tests and head-up tilt (Table 4.2). In controls,
the FBF increased during MA and fell during tilt at 2 and 10 minutes with reciprocal changes in FVR (Table 4.3.). In MS patients, changes in FBF during pressor stimuli were not significant, however, the FBF fell during tilt with a reciprocal rise in VR (Table 4.3.). In controls, the SMBF fell during MA, CC, ISE and tilt (Table 4.3.). There was a reciprocal increase in VR (Table 4.3.). In MS patients as a group, there were no significant changes in SMABF during pressor tests with a fall during tilt (Table 4.3. and Fig 4.7b.). There were no changes in VR during pressor tests but a rise during tilt (Table 4.3.). The Valsalva ratio was similar in controls and MS patients (1.7±0.17 and 1.6±0.14 respectively. The response to DB was similar in controls (18±1.8) and in MS patients (17±1.5).
Table 4.2. Average systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial blood pressure (MABP), heart rate (HR), and cardiac index (CI) before (Pre) and during mental arithmetic (MA), cutaneous cold (CC), isometric exercise (ISE) and tilt at 2 (Tilt 2) and 10 (Tilt 10) minutes in normal controls (C) and MS patients (MS).

<table>
<thead>
<tr>
<th></th>
<th>Pre.MA</th>
<th>MA</th>
<th>Pre.CC</th>
<th>CC</th>
<th>Pre.ISE</th>
<th>ISE</th>
<th>Pre.Tilt</th>
<th>Tilt 2</th>
<th>Tilt 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP mmHg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>129±4</td>
<td>146±5*</td>
<td>128±5</td>
<td>144±5*</td>
<td>131±5</td>
<td>156±8*</td>
<td>130±4</td>
<td>127±4</td>
<td>127±5</td>
</tr>
<tr>
<td>MS</td>
<td>121±7</td>
<td>129±7</td>
<td>119±5</td>
<td>135±7</td>
<td>129±7</td>
<td>125±6</td>
<td>121±6</td>
<td>124±6</td>
<td></td>
</tr>
<tr>
<td>DBP mmHg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>76±3</td>
<td>83±3</td>
<td>73±3</td>
<td>83±3*</td>
<td>76±4</td>
<td>91±8</td>
<td>73±2</td>
<td>79±3</td>
<td>78±3</td>
</tr>
<tr>
<td>MS</td>
<td>72±5</td>
<td>75±4</td>
<td>71±3</td>
<td>77±4</td>
<td>73±5</td>
<td>74±6</td>
<td>71±4</td>
<td>77±5</td>
<td>75±4</td>
</tr>
<tr>
<td>MABP mmHg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>94±3</td>
<td>104±3*</td>
<td>92±3</td>
<td>103±4*</td>
<td>94±2</td>
<td>113±7*</td>
<td>92±2</td>
<td>95±3</td>
<td>95±3</td>
</tr>
<tr>
<td>MS</td>
<td>87±5</td>
<td>93±5</td>
<td>86±4</td>
<td>97±5</td>
<td>87±4</td>
<td>92±6</td>
<td>89±4</td>
<td>92±5</td>
<td>91±4</td>
</tr>
<tr>
<td>HR Beats/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>73±3</td>
<td>78±4</td>
<td>71±3</td>
<td>78±4</td>
<td>69±4</td>
<td>81±3*</td>
<td>71±3</td>
<td>79±4</td>
<td>78±4</td>
</tr>
<tr>
<td>MS</td>
<td>63±3</td>
<td>67±3</td>
<td>57±2</td>
<td>61±3</td>
<td>59±3</td>
<td>62±2</td>
<td>61±3</td>
<td>69±4</td>
<td>72±3*</td>
</tr>
<tr>
<td>CI Units</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>686±56</td>
<td>710±36</td>
<td>632±74</td>
<td>710±91</td>
<td>563±70</td>
<td>741±77</td>
<td>695±40</td>
<td>531±69</td>
<td>540±63*</td>
</tr>
<tr>
<td>MS</td>
<td>591±48</td>
<td>556±46</td>
<td>495±40</td>
<td>480±50</td>
<td>504±44</td>
<td>614±54</td>
<td>502±45</td>
<td>460±53</td>
<td>437±34</td>
</tr>
</tbody>
</table>

*p<0.05
Table 4.3. Superior mesenteric artery blood flow (SMABF), superior vascular resistance (SMAVR), forearm blood flow (FBF) and forearm vascular resistance (FVR) before (Pre) and during mental arithmetic (MA), cutaneous cold (CC), isometric exercise (ISE) and tilt at 2 (Tilt 2) and 10 (Tilt 10) minutes in normal controls (C) and MS patients (MS).

<table>
<thead>
<tr>
<th></th>
<th>Pre.MA</th>
<th>MA</th>
<th>Pre.CC</th>
<th>CC</th>
<th>Pre.ISE</th>
<th>ISE</th>
<th>Pre.Tilt</th>
<th>Tilt 2</th>
<th>Tilt 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMABF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>469±55</td>
<td>315±43*</td>
<td>533±63</td>
<td>316±43*</td>
<td>553±45</td>
<td>323±32*</td>
<td>509±55</td>
<td>291±41*</td>
<td>282±38*</td>
</tr>
<tr>
<td>MS</td>
<td>471±66</td>
<td>339±34</td>
<td>418±65</td>
<td>322±55</td>
<td>425±58</td>
<td>307±49</td>
<td>468±71</td>
<td>236±32*</td>
<td>239±22*</td>
</tr>
<tr>
<td>SMAVR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.22±0.03</td>
<td>0.4±0.06*</td>
<td>0.2±0.02</td>
<td>0.4±0.06*</td>
<td>0.2±0.03</td>
<td>0.5±0.13*</td>
<td>0.2±0.02</td>
<td>0.4±0.06*</td>
<td>0.4±0.07*</td>
</tr>
<tr>
<td>MS</td>
<td>0.21±0.02</td>
<td>0.28±0.03</td>
<td>0.24±0.03</td>
<td>0.31±0.04</td>
<td>0.23±0.03</td>
<td>0.3±0.04</td>
<td>0.2±0.03</td>
<td>0.43±0.05*</td>
<td>0.4±0.04*</td>
</tr>
<tr>
<td>FBF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>2.7±0.2</td>
<td>4±0.5*</td>
<td>2.6±0.3</td>
<td>2.3±0.3</td>
<td>2.5±0.3</td>
<td>3.4±0.4</td>
<td>2.7±0.26</td>
<td>1.5±0.2*</td>
<td>1.5±0.3*</td>
</tr>
<tr>
<td>MS</td>
<td>3±0.4</td>
<td>3.4±0.7</td>
<td>3±0.4</td>
<td>2.3±0.3</td>
<td>2.8±0.4</td>
<td>4.4±0.7</td>
<td>3±0.4</td>
<td>1.8±0.2*</td>
<td>2±0.3*</td>
</tr>
<tr>
<td>FVR Units</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>37±3</td>
<td>29±3.5</td>
<td>37±3</td>
<td>60±12</td>
<td>41±4.3</td>
<td>35±3.5</td>
<td>37±3</td>
<td>74±8*</td>
<td>74±8*</td>
</tr>
<tr>
<td>MS</td>
<td>33±4</td>
<td>39±7</td>
<td>33±5</td>
<td>49±7</td>
<td>36±5</td>
<td>24±3</td>
<td>35±5</td>
<td>53±7*</td>
<td>55±7*</td>
</tr>
</tbody>
</table>

*p<0.05
Fig 4.7. SMABF changes to Pressor Stimuli in MS.
(a) Individual changes in mean arterial blood pressure (ΔMABP) and (b) individual changes is superior mesenteric artery blood flow (ΔSMABF) during mental arithmetic (MA) (upper panel), cutaneous cold (CC) (middle panel) and isometric exercise (ISE) (lower panel) in 10 patients with multiple sclerosis. The average changes in MABP in controls (C) during MA, CC, and ISE are shown separately on the right. The bars indicate ±s.e.m.

This study has demonstrated that there were impaired responses to pressor stimuli in six out of ten patients with secondary progressive MS who had increased PBMC β-adrenoceptor densities. None of these patients, however, had postural hypotension—a cardinal sign which when not present, is often thought to exclude significant autonomic dysfunction. In none of the MS patients was there evidence of parasympathetic involvement of cardiovascular pathways.

These observations differ from those previously described in which additional abnormalities noticed in the cardiovascular responses to DB, VM, and the BP response to standing (Mutani et al., 1982; Neubauer et al., 1978; Nordenlo et al., 1989; Pentland et al., 1987; Senaratne et al., 1982; Sternman et al., 1985). This could be owing to a variety of reasons. Of importance is the clinical state of the patients. This can vary considerably, depending upon the duration and form of the disease. In our patients, with a high disability score and an extensive and progressive form of the disease, the lack of postural hypotension and normal responses to the VM indicated overall preservation of the baro-receptor reflex pathways. These tests depend on activation of both afferent and efferent pathways, both of which appeared to be functioning normally. The HR responses to deep breathing were normal, confirming further the integrity of cardiac parasympathetic function in all our patients. The latter
observations differ from previous studies (Pentland et al., 1987; Senaratne et al. 1985), where varying autonomic abnormalities or technical factors may have accounted for these differences.

The normal responses to head-up tilt and to the VM were suggestive of intact sympathetic nerve pathways. This contrasted with the responses to the three pressor tests—MA, CC and ISE, which were abnormal in six out of the ten MS patients. Two of these tests depend upon activation of sympathetic efferent pathways, in response to stimulation of sensory afferents, in skin with CC and in muscle during ISE. In addition, there is a strong central component to both responses, and this is particularly so in relation to the autonomic responses to MA. The lack of response extended not only to systemic pressor changes but also to regional responses, especially in the large splanchnic vascular territory. Measurement of changes in SMBF reflect changes in this vascular bed and in normal subjects there is vasoconstriction to such stimuli, consistent with its rich sympathetic innervation (Chaudhuri et al., 1991; Chaudhuri et al., 1992a). In patients with primary autonomic failure and definite sympathetic impairment, these changes do not occur (Chaudhuri et al., 1992a). No changes were observed in those patients in whom there was no pressor response. This is unlikely to have been due to efferent sympathetic failure as the superior mesenteric vascular bed was capable of response during head-up tilt,
where the fall in SMBF and the rise in vascular resistance confirmed the ability of such patients to constrict actively this vessel, as in normal subjects. The lack of a response to these three pressor stimuli in six of the MS patients, in the presence of functional sympathetic efferent pathways, was suggestive of a lesion in other segments of the baroreflex pathway. Impairment of afferent pathways either from the skin or from skeletal muscle seemed unlikely, especially as there was no evidence of a sensory nerve deficit. Furthermore, an afferent lesion alone would not explain the lack of pressor response to cerebral stimulation by MA. This suggested that the common site of the abnormality was likely to be within the cerebral centres and pathways, converging on to those brain regions, which result in sympathetic efferent activation, such as the brain stem. This could involve the spinal cord, but this is less likely in view of the preserved responses to tilt. The observations are consistent with the diffuse inflammatory processes in the central nervous system, which are known to involve large sections of white matter in MS. Attempts were therefore made to identify and relate the anatomical sites at which lesions were noted on MRI, but there were difficulties because of the widespread distribution of the lesions, especially as precise knowledge of the putative pathways and centres involved in the cerebral processing of these stimuli is not available. Furthermore, the relatively low resolution of the scans may not have identified minute but
functionally significant lesions. Although in this study we have reported on a small number of MS patients, the present observations lend themselves to consideration, ideally in larger numbers, of an in-depth analysis of specific autonomic abnormalities and their correlation with lesions using higher-resolution MRI. Our inability to correlate the two in this intensive but small study, is consistent with previous studies where attempts to relate cognitive function with precisely localized anatomical deficits on MRI in MS have not been successful, despite demonstration of a positive correlation between cognitive impairment and the density of lesions (Callaman et al., 1989).

This study, therefore, indicates that despite the absence of postural hypotension, and in the presence of a normal VM and other indices of cardiac parasympathetic function, there may be selective involvement of autonomic function, especially in relation to pressor tests which are processed through various cerebral centres before activating sympathetic efferent pathways. These abnormalities relate to the systemic pressor response and to the regional vascular response in the splanchnic bed. They do not appear to be due to impairment of sympathetic efferent activity and are more readily explained by patchy and multifocal abnormalities within the brain, probably involving the neurons concerned with motor, sensory and higher function and their interconnections with brain stem cardiovascular centres.
4.3. Assessment of central autonomic function in Multiple Sclerosis: cardiovascular and neurohormonal responses to clonidine.

Autonomic nervous system abnormalities documented by physiological assessment in progressive MS patients who had increased β-adrenoceptor densities on PBMCs, have indicated a central autonomic dysfunction which has been attributed to inflammatory lesions involving autonomic centres and pathways (Thomaides et al., 1993b). However, there have been no pharmacological studies with drugs which may serve as central neurophysiological probes. Clonidine is an alpha-2 adrenoceptor agonist that reduces blood pressure (Reid et al., 1977; Reid., 1981), lowers plasma NA (Reid, 1981) and insulin (Barbieri et al., 1980), and increases plasma glucose and GH (May et al., 1990; Gil-Ad et al., 1979; Lal et al., 1981; Hunt et al., 1986). Clonidine has been used in well defined models of autonomic impairment such as tetraplegia (Reid et al., 1977) and pure autonomic failure (Thomaides et al., 1993a). In a more recent study in pure autonomic failure patients (PAF) and patients with multiple system atrophy (MSA, Shy-Drager syndrome) distinctive neurohormonal responses to clonidine has been reported (Thomaides et al., 1993a). However, there are no studies of haemodynamic and neurohormonal responses to clonidine in MS patients.

In an attempt to evaluate the extent of central
autonomic dysfunction in progressive MS patients and compare those with dysfunction observed in different autonomic failure groups of patients, responses to clonidine in secondary progressive MS patients, PAF patients, MSA; Shy-Drager syndrome patients and normal subjects have been investigated.

4.3.1. Methods and subjects.

In this study, 10 MS patients (4 male and 6 female with mean age 42±11) with secondary progressive form of disease (Thompson et al., 1992), who had undergone physiological and haemodynamic function studies, 10 patients with pure autonomic failure (PAF) (5 males and 5 females, mean age 59.8±5.8), 10 patients with multiple system atrophy (MSA; Shy-Drager syndrome) (6 males and 4 females mean age 57.8±10.1) and 10 healthy normal subjects (NS) (4 males and 6 females mean age 42±11, range of age 23-72 years) took part. MSA patients had no levodopa or other anti-parkinsonian medication for at least 3 months before the study. PAF and MSA patients had not received flurocortisone treatment for at least 4 days prior to investigation. The clinical criteria for selection were: 1) for the NS: normal autonomic function physiological tests, 2) for the PAF patients: postural hypotension over 20 mm Hg of systolic blood pressure, low plasma NA levels, sympathetic vasoconstrictor failure and sudomotor and urinary bladder impairment and 3) for the MSA patients:
akinetic rigid syndrome, autonomic failure, postural hypotension over 20 mm Hg of systolic blood pressure, absent plasma noradrenaline (NA) response to head-up tilt, sympathetic vasoconstrictor failure, sudomotor and urinary bladder impairment. All subjects were studied after an overnight fast at 09:30 hours, in a temperature controlled room (22°C) after 30 minutes supine rest, following familiarisation with equipment. An IV cannula (Abbocath-T, 18G) was inserted into a forearm vein for blood collection and clonidine infusion. Measurements were then made, before and 15, 30, 45 and 60 minutes after clonidine infusion (2µg/kg IV over 10 minutes). BP and HR (automated sphygmomanometer, Sentron), CI, continuous wave ultrasound, Exerdop), index finger and body skin temperature (multiple thermistors, Panlab), FBF, strain gauge plethysmography), SDBF, laser doppler flowmetry, Periflux PF 2B) and SMABF, pulse doppler flowmetry, Acuson 128) (Chaudhui et al., 1991), were measured noninvasively. SMAVR and FVR were calculated by dividing the mean arterial blood pressure by flow. Autonomic failure patients were provided by Prof. C.J. Mathias. All studies were carried out by Dr Y. Zoukos and T. Thomaides.

4.3.2. Measurements of plasma levels of noradrenaline (NA) and adrenaline (Ad).

Blood (2.5 ml) for measurements of noradrenaline and adrenaline was collected into cooled tubes containing 20µl
of ethyleneglycolbis(aminooethyl ether)tetra-acetate (0.095% w/v)/glutathione (0.06%w/v) per 1 ml of blood. Plasma was stored at -70°C until assay. Catecholamines were extracted from plasma using the solvent extraction method described by Smedes. Separation was achieved by high-performance liquid chromatography (HPLC) on a 5 μm 22cmX0.46 cm ODS column (RP 18, Brownlee Labs) using 15% (v/v) acetonitrile in 0.05 mol/l phosphate/acetate buffer pH 3.0, containing sodium dodecyl sulphate (120 mg/l) as the mobile phase. Catecholamines were detected by electrochemical detection (ESA Coulomcho 5100 A) with the conditioning cell (5021) set at +0.04 V and the analytical cell (5011) at -0.10 V for detector and -0.35 V for detector 2. Using 1 ml samples of rabbit plasma, the inter-assay coefficient of variation for noradrenaline was 6.6% (2.42±0.05 nmol/l, mean±sem, n=10) and for adrenaline was 11.0% (0.31±0.01 nmol/l, mean±sem, n=10). The detection limit when using 1 ml of plasma was 0.1 nmol/l. Fig 4.8. shows chromatograms of extracts of a standard mixture and a plasma sample. All measurements were carried out by Mr D. Pavitt at the Cardiovascular Unit of St Mary's Hospital School of Medicine Laboratories.

4.3.3. Growth hormone (GH) measurements.

Plasma from heparinized blood samples was aliquotted at -20°C. GH was measured using 125I-labelled GH radioimmunoassay kit (NERTIA); the results were calculated
and levels expressed in mU/l. The intra-assay and inter-assay coefficients of variance were 9 and 10% respectively.

Fig 4.8. HPLC Chromatograms of catecholamines.
Chromatograms of extracts of a standard solution (a) and a plasma sample (b) obtained with solvent extraction and HPLC with electrochemical detection. (a) containing 70 ng of noradrenaline (NA), 76 ng of adrenaline (Ad), 60 ng of dihydroxybenzylamine (DHBA), and 127 ng of dopamine (DA) per ml; (b) containing 515 pg of NA, 125 pg of Ad, 600 pg of DHBA per ml. (Provided by Mr D. Pavitt, Pickering Unit, St. Mary's Hospital, School of Medicine.)
4.3.4. Statistical analysis.

Changes in SMABF were expressed as percentage changes from baseline values. Statistical analysis was performed using Student’s paired and unpaired t-tests for absolute values and analysis of variance (ANOVA). P<0.05 was considered significant, and data are presented as means±s.e.m.

4.3.5. Results.

4.3.6. Systemic blood pressure (BP) and heart rate (HR).

In control subjects, supine mean arterial blood pressure (MABP) \([\text{MABP}=\text{SBP}+2\text{DBP}/3]\) was 91±2.1 mm Hg and fell to 80±2.5, 77±2.1, 76±2.5 and 76±2.3 at 15, 30, 45 and 60 min after clonidine respectively (each p<0.05, Fig 4.9.). In patients with MSA, supine MABP was 111±4.8 mm Hg and after clonidine it fell to 92±5.1, 84±4.8, 86±4.6 and 86±5.4 at 15, 30, 45 and 60 min, respectively (each p<0.05, Fig 4.9.). In the patients with PAF, supine MABP was higher (113±6.5 mm Hg) than in the control subjects (91±2.1 mm Hg) and remained unchanged after clonidine (Fig 4.9.).

In MS patients, supine MABP was 85±4 and after clonidine there was not a significant fall at 15, 30, 45 and 60 min (78±4, 79±5, 81±4.3, and 81±5.1 respectively for each time point). Individual analysis of the results
Fig 4.9. MABP after clonidine in NS, MSA and PAF.

Changes in MABP before and after clonidine in control subjects (●-●), patients with MSA (■-■) and patients with PAF (▲-▲). Values are means with bars indicating s.e.m. Statistical significance *p<0.05.
showed however that in 5 MS patients there was a fall in MABP after clonidine (Fig 4.10.). According to the BP response after clonidine, we subdivided the MS patients in two groups the MS-responders (MSR) and the MS-non-responders (MSNR). Changes in HR after clonidine in control subjects, patients with MSA, PAF and MS were not significant (Table 4.4.).

4.3.7. Regional haemodynamic measurements.

In control subjects, resting SMABF was 445±35 ml/min and rose by a maximum of 22.5±5% (445±35 to 538±41 ml/min at 30 min) after clonidine. In patients with MSA, resting SMABF was 433±52 ml/min (similar to control subjects) and rose by a maximum of 38.8±14% (433±52 to 574±68 ml/min at 30 min) after clonidine (Fig 4.11.a). Resting SMABF was higher in patients with PAF (601±62 ml/min) compared with patients with MSA, control subjects and MS patients (p<0.05) and was unchanged after clonidine (601±62 to 630±60 ml/min, 4.5%; not significant Fig 4.11.a). In MSR patients resting SMABF was 380±80 ml/min and rose by a maximum 26±2% (380±80 to 480±60 ml/min) 60 min after clonidine (Fig 4.12.a). However, in MSNR patients the resting SMABF was 360±40 and remained unchanged after clonidine (Fig 4.12.a). Changes in SMABF in control subjects and patients with MSA and MSR were significantly different from those in patients with PAF and MSNR (p<0.05). There was a corresponding fall in SMAVR after
clonidine by 30.6±3.3% (0.22±0.02 to 0.15±0.02 units at 45 min) in control subjects and by 43±6.3% (0.3±0.05 to 0.17±0.03 at 45 min) in patients with MSA (each p<0.05, Fig 4.11.) In MSR group of patients there was a fall of SMAVR by 27±3.1% (0.26±0.04 to 0.17±0.2, 0.17±0.02 units at 45 and 60 min respectively, each p<0.05 Fig 4.12.). SMAVR remained unchanged in patients with PAF and MSNR after clonidine.

In control subjects and patients with MSA and MS after clonidine FBF fell. In PAF patients however there was no change in FBF after clonidine. (Table 4.5.).

Index finger temperature (FT) remained unchanged after clonidine in patients with MSA and PAF. FT rose in control subjects at 30, 45 and 60 minutes and in MS patients at 30 and 45 minutes. There was a trend towards a higher skin digital blood flow after clonidine at 30 min in control subjects and MSA patients and 45 and 60 min in MS patients. Basal levels of NA and Ad were similar to the control subjects (NA;486±136, Ad;46±7 pg/ml) in MSA (NA;433±88 pg/ml, Ad;37±6 pg/ml) and MS patients (NA;433±88 pg/ml, Ad;40±11 pg/ml) but low in PAF (NA;90±37 pg/ml, Ad;23±5 pg/ml). After clonidine, a significant fall was observed in all studied groups except in PAF (Table 4.6.).
Fig 4.10. MABP after clonidine in NC and MS patients.

Changes in MABP before and after clonidine in 10 controls (C) (●-●), 5 responsive (MSR) (●—●) and 5 non-responsive (MSNR) (▲-▲) MS patients. Values are means with bars indicating s.e.m. Statistical significance: *p<0.05.
Table 4.4. Changes in mean arterial blood pressure (MABP), heart rate (HR) and cardiac index (CI) before and after clonidine in control subjects and patients with MSA, PAF and MS.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean arterial blood pressure (MABP) mm Hg</td>
<td>91±2.1</td>
<td>80±2.5*</td>
<td>77±2.1*</td>
<td>76±2.5*</td>
<td>76±2.3*</td>
</tr>
<tr>
<td>MSA</td>
<td>111±4.8</td>
<td>92±5.1*</td>
<td>84±4.8*</td>
<td>86±4.6*</td>
<td>86±5.4*</td>
</tr>
<tr>
<td>PAF</td>
<td>113±6.5+</td>
<td>114±7</td>
<td>108±8</td>
<td>106±9</td>
<td>106±7.4</td>
</tr>
<tr>
<td>MS</td>
<td>85±4</td>
<td>78±4</td>
<td>79±5</td>
<td>81±4</td>
<td>81±5</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate (HR) (beats/min)</td>
<td>67.9±2.6</td>
<td>66.1±2.3</td>
<td>65.9±2.1</td>
<td>66.3±2.2</td>
<td>65.1±1.9</td>
</tr>
<tr>
<td>MSA</td>
<td>75.1±3.1</td>
<td>74.1±3.7</td>
<td>73±4.1</td>
<td>74.8±4</td>
<td>73.5±3.5</td>
</tr>
<tr>
<td>PAF</td>
<td>69.5±2.6</td>
<td>66.1±2.4</td>
<td>64±2.8</td>
<td>66.6±2.6</td>
<td>66.6±2.5</td>
</tr>
<tr>
<td>MS</td>
<td>66±5.2</td>
<td>65±5.9</td>
<td>63±5.1</td>
<td>65±7</td>
<td>64.2±4.8</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiac index (units)</td>
<td>775±81</td>
<td>713±78</td>
<td>645±66</td>
<td>652±65</td>
<td>673±72</td>
</tr>
<tr>
<td>MSA</td>
<td>709±47</td>
<td>577±43*</td>
<td>560±47*</td>
<td>543±45*</td>
<td>571±52</td>
</tr>
<tr>
<td>PAF</td>
<td>583±67</td>
<td>567±68</td>
<td>548±64</td>
<td>517±59</td>
<td>496±63</td>
</tr>
<tr>
<td>MS</td>
<td>561±71.2</td>
<td>478±67</td>
<td>469±69</td>
<td>427±49</td>
<td>462±57</td>
</tr>
</tbody>
</table>

*p<0.05 t-student paired test and +p<0.05 ANOVA test.
Fig 4.11. SMABF and SMAVR after clonidine in NC, MSA and PAF.

Changes in SMABF (a) and SMAVR (b) before and after clonidine in control subjects (●-●), and patients with MSA (■-■) and PAF (▲-▲). The fall in SMAVR was significant (p<0.05) in control subjects and patients with MSA at 30 and 45 min, with no change in patients with PAF. Values are means with bars indicating ±s.e.m.
Fig 4.12. SMABF and SMAVR in NC and MS.

Changes in SMABF (a) and SMAVR (b) before and after clonidine in 10 controls (C), 5 responsive (MSR) and 5 non-responsive (MSNR) MS patients.*p<0.05.
Table 4.5. Changes in forearm blood flow (FBF), skin digital blood flow (SDF) and finger temperature (FT) before and after clonidine in controls (C) and patients with MSA, PAF and MS.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FOREARM BLOOD FLOW</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.09±0.32</td>
<td>2.65±0.26</td>
<td>2.08±0.22*</td>
<td>2.07±0.23*</td>
<td>2.56±0.27</td>
</tr>
<tr>
<td>MSA</td>
<td>3.08±0.33</td>
<td>2.42±0.29</td>
<td>2.13±0.28</td>
<td>2.13±0.28</td>
<td>2.52±0.32</td>
</tr>
<tr>
<td>PAF</td>
<td>2.53±0.23</td>
<td>2.37±0.26</td>
<td>2.25±0.28</td>
<td>2.25±0.28</td>
<td>2.15±0.26</td>
</tr>
<tr>
<td>MS</td>
<td>3.63±0.56</td>
<td>3.12±0.51</td>
<td>2.87±0.49</td>
<td>2.84±0.53</td>
<td>3.31±0.33</td>
</tr>
<tr>
<td><strong>SKIN DIGITAL BLOOD FLOW</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>44±4.6</td>
<td>51±4.4</td>
<td>56.1±4.3</td>
<td>54.9±4.7</td>
<td>54.6±4.3</td>
</tr>
<tr>
<td>MSA</td>
<td>43±9.5</td>
<td>41.9±7.1</td>
<td>49.8±9.5</td>
<td>54.8±11</td>
<td>52.6±11</td>
</tr>
<tr>
<td>PAF</td>
<td>49±7.6</td>
<td>42±5.5</td>
<td>42.2±7.7</td>
<td>48.7±6</td>
<td>48.6±6</td>
</tr>
<tr>
<td>MS</td>
<td>38.5±5.2</td>
<td>58±13.5</td>
<td>62.5±11.8</td>
<td>79.4±13</td>
<td>70±10.8</td>
</tr>
<tr>
<td><strong>FINGER TEMPERATURE (°C)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>31.5±0.8</td>
<td>32.9±0.5</td>
<td>33.6±0.43*</td>
<td>33.5±0.5*</td>
<td>33.6±0.5*</td>
</tr>
<tr>
<td>MSA</td>
<td>28.5±1.9</td>
<td>30.55±1.9</td>
<td>31.22±1.7</td>
<td>31.09±1.8</td>
<td>30.95±1.8</td>
</tr>
<tr>
<td>PAF</td>
<td>30.03±1.2</td>
<td>30.38±1.2</td>
<td>31.58±0.96</td>
<td>31.88±0.97</td>
<td>31.99±0.1</td>
</tr>
<tr>
<td>MS</td>
<td>31.6±1.2</td>
<td>33±0.70</td>
<td>34.2±0.48*</td>
<td>34.3±0.48*</td>
<td>33.9±0.51</td>
</tr>
</tbody>
</table>

*p<0.05 t-student paired test.
Fig 4.13. MABP and GH changes after clonidine in MS.

Maximum individual changes in mean arterial blood pressure (ΔMABP) and growth hormone (GH) in 5 responsive (MSR, stippled histograms) and 5 non-responsive (MSNR, hatched histograms) MS patients. Average changes in controls (C) is indicated on the right.
Table 4.6. Plasma levels of noradrenaline (NA), adrenaline (Ad) and growth hormone (GH) before and after clonidine in controls (C) and patients with MSA, PAF and MS.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>486±136</td>
<td>365±118</td>
<td>339±108</td>
<td>298±89*</td>
<td>383±118</td>
</tr>
<tr>
<td>MSA</td>
<td>433±88</td>
<td>285±108</td>
<td>285±111</td>
<td>273±82*</td>
<td>284±106</td>
</tr>
<tr>
<td>PAF</td>
<td>90±37+</td>
<td>77±26</td>
<td>63±20</td>
<td>59±19</td>
<td>66±19</td>
</tr>
<tr>
<td>MS</td>
<td>568±130</td>
<td>456±124*</td>
<td>447±123*</td>
<td>506±144</td>
<td>482±134</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>46±7</td>
<td>38±7</td>
<td>28±7</td>
<td>33±7*</td>
<td>30±4*</td>
</tr>
<tr>
<td>MSA</td>
<td>37±6</td>
<td>31±6</td>
<td>26±4*</td>
<td>23±3*</td>
<td>24±2*</td>
</tr>
<tr>
<td><strong>ADRENALINE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>2.78±1.8</td>
<td>5.06±1.5</td>
<td>7.17±3.1</td>
<td>12.9±5*</td>
<td>15.7±5*</td>
</tr>
<tr>
<td>MSA</td>
<td>5.49±1.8</td>
<td>4.3±1.3</td>
<td>3.5±0.7</td>
<td>4.9±1.8</td>
<td>3.7±1.2</td>
</tr>
<tr>
<td><strong>GROWTH HORMONE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>2.51±1.7</td>
<td>5.85±2.6</td>
<td>16.4±6.5*</td>
<td>19.4±7*</td>
<td>22±9*</td>
</tr>
<tr>
<td>PAF</td>
<td>6.83±2.6+</td>
<td>10.73±5</td>
<td>14.8±8</td>
<td>15±8.5</td>
<td>9±5</td>
</tr>
</tbody>
</table>

*p<0.05 t-student paired test, *p<0.05 ANOVA test
Basal GH levels were higher compared to control subjects (GH; 2.78±1.8 mU/l) in MS patients (GH; 6.83±2.6 mU/l). After clonidine an increase in GH was observed in control subjects at 45 and 60 min, and in PAF patients at 30, 45, 60 min but not in MS and MSA patients (Table 4.6.). Individual analysis of the responses revealed no change after clonidine in 8 out of the 10 MS patients (Fig 4.13.).

4.3.8. Discussion.

In controls, after clonidine, there was a fall in blood pressure, SMABF tended to rise and SMAVR fell, indicating active dilatation of this artery which is a major constituent of the splanchnic vascular bed. This is consistent with previous data indicating that changes in SMABF play an important role in blood pressure control in normal man (Chaudhuri et al., 1991), while the lack of change in SMABF may contribute to the abnormal blood pressure regulations in human autonomic failure (Chaudhuri et al., 1992a).

In patients with chronic autonomic failure, clonidine causes differential responses to BP. As in control subjects there is a reduction in BP in MSA, which differs from the lack of fall in BP occurring in PAF. Both the patients with MSA and PAF had clear evidence of sympathetic failure, as based on a combination of
physiological tests and on the impaired response of plasma NA levels to head-up tilt, when their BP fell. There were however, differences in the basal plasma levels of NA between the two autonomic failure patient groups, these being within the normal range in the majority of the patients with MSA, whereas this is the reverse in patients with PAF, in whom the levels were subnormal. In conjunction with work from different sources (Kooner et al., 1989; Polinsky, 1992), this suggests sympathetic failure caused by a more central lesion in MSA and by a more peripheral lesion in PAF.

The different pressor responses to clonidine in the two groups of autonomic failure have raised the possibility of different effects of clonidine upon a major vascular bed, such as the splanchnic region. Using the Doppler technique for the measurements of SMABF it has been shown that the basal levels of SMABF in MSA patients were similar to the control subjects, levels in patients with PAF, however, were higher. The precise reasons for this are unclear. A greater degree of peripheral denervation in PAF may have contributed. The calculated SMAVR, however, was similar in patients with PAF and control subjects, but considerably higher in patients with MSA. This may reflect a tendency in patients with MSA to greater constriction within this vascular bed.

After clonidine, SMABF rose in the control subjects
and in patients with MSA, but not in patients with PAF. Reciprocal changes were reflected in calculated SMAVR. In control subjects, this suggests withdrawal of neural sympathetic tone to the vascular bed. The response to clonidine in patients with MSA suggested that their basal sympathetic outflow was within the normal limits. However, they had impaired autonomic responses to a range of stimuli which are dependent on the integrity of sympathetic pathways, and the apparently 'normal' basal tone may indicate partial preservation of sympathetic pathways, particularly in the presence of pressor supersensitivity (Williams et al., 1986). In patients with PAF, however, after clonidine, there were no changes in SMABF or in SMAVR. This is consistent with peripheral sympathetic denervation and the inability of clonidine to further reduce low sympathoneural tone. In patients with PAF, the SMA may have been dilated maximally before clonidine, with further dilatation therefore not possible.

Although previously described, the changes in cardiac function and in other regional beds bear mention, particularly in relation to the differences in the two groups with primary autonomic failure (Thomaides et al., 1992), in neither group, as in the control subjects, was there a significant change in HR. CI, however, fell in patients with MSA and to a lesser extent in the control subjects; in patients with MSA this suggests a contribution of residual sympathoneural activity to
cardiac function. This did not occur in patients with PAF. In neither patients with MSA nor patients with PAF were there changes indicating active responses in the forearm muscle vasculature, excluding significant effects upon the skeletal muscle bed. Digital skin blood flow did not significantly increase in patients with MSA and control subjects and was unchanged in patients with PAF. Finger index temperature rose significantly in control subjects only. This is consistent with the ability, albeit partial in patients with MSA, to withdraw vascular tone in this vascular bed which is richly innervated by the sympathetic nervous system (Kooner et al., 1991); this did not occur in PAF.

In MS patients there was a variable response to clonidine. In 5 of the 10 patients, there was a fall in BP, with a trend towards an increased SMABF, a fall in SMAVR and an increase in index skin finger temperature. This was similar to the responses in normal subjects. In the other 5 patients no change in the BP after clonidine was reported. This group which was called MSNR also did not demonstrate an increase in SMABF with reciprocal reduction of SMAVR. However, the basal levels of SMABF were within the normal limits unlike in PAF in whom an increase was observed probably indicating denervation. In MSNR also index skin finger temperature did not increase after clonidine. Cardiac function indicators as HR and CI remained unchanged throughout the clonidine test in both
responder and non-responder MS patients. Although in MSNR patients the effects of clonidine upon BP and other parameters indicate a similar response as in PAF patients the cause of this is unlikely to represent similar pathology in both group of patients for a number of reasons. Abnormal responses to clonidine in PAF seem to be due to the peripheral dysfunction of sympathetic function as the presence of postural hypotension, low levels of plasma NA and high SMABF indicate. In MSNR, however, there was no evidence of severe peripheral autonomic lesion as based on the lack of postural hypotension, normal VM and the normal plasma noradrenaline levels. Abnormal responses to clonidine could be attributed to lesions interrupting descending sympathetic pathways as has been described in patients with cervical spinal cord transection (Reid et al., 1977). However, in the MSNR patients normal responses to physiological tests, other than the pressor tests, excluded significant lesions in the descending sympathetic efferent pathways within the brain stem/or spinal cord. This would suggest therefore that in 5 out of the 10 MS patients there were lesions involving either those brain stem nuclei responsible for activation of the sympathetic efferent pathways or, as was more likely and as based on the responses to other tests, those cerebral areas or pathways in communication with brain stem centres which modulate sympathetic efferent pathways. This would be consistent with the widespread lesions, seen in the white matter of patients with MS.
Neurohormonal studies after clonidine, revealed pronounced differences in the GH response in PAF as compared to MSA patients (Thomaides et al., 1993a). After clonidine, increased release of GH has been noticed in PAF patients and normal subjects but not in MSA patients. It seems likely that the impaired GH response to clonidine in MSA results from abnormalities in the neuronal system and/or pathways from the hypothalamus that are concerned with secretion of GH. In healthy human beings, alpha adrenoceptor activation stimulates GHRH or suppresses somatostatin (Alba Roth et al., 1989; Muller et al., 1988). The opioid and cholinergic systems, which are linked with the noradrenergic system (May et al., 1988), may also influence GH release (Bramnert et al., 1984; Delitala et al., 1983). This study suggests that there is sparing of these central systems and pathways in PAF, but not in MSA. This notion is consistent with previous data which point to lesions in the spinal cord and/or periphery in PAF, but to lesions within the hypothalamus and brain stem in MSA (Polinsky et al., 1992). More interestingly, in 8 out of the 10 MS patients studied there were impaired GH responses after clonidine. As impaired release of GH is known to occur in elderly patients, this seems unlikely to account for the responses in MS patients, since comparisons were made with both age and sex matched subjects (Rudman et al., 1981). Also, impaired responses have been noted in endogenous depression (Checkley et al., 1981). Although formal Hamilton score ratings were not
obtained, none of our patients were clinically depressed. It seems more probable therefore that these patients had lesions in the hypothalamus or the central pathways concerned with growth hormone release (Mazza et al., 1990). This would be consistent with experimental observations following intraventricular infusions of clonidine, that it acts at periventricular sites to increase GH secretion (Laborit et al., 1990). There are usually a number of lesions in these areas in patients with MS, as was also noted in our patients, although no clear MRI differences between those with normal and abnormal responses could be specifically delineated.

Our studies therefore indicate that with clonidine we can differentiate two sets of abnormal responses in patients with secondary progressive MS. In 5 out of 10 subjects cardiovascular responses were impaired, suggestive of central involvement in relation to cerebral centres and interconnections with brain stem cardiovascular centres. In 8 out of 10 patients there were abnormal GH responses, suggestive of lesions within the hypothalamus and its connections to the pituitary gland. The dissociation between the cardiovascular and GH responses, is consistent with the patchy nature of the lesions in MS.
4.4. Expression of $\beta$-adrenoceptors on PBMCs before and after reduction of central sympathetic outflow by clonidine in normal subjects and in patients with Autonomic failure or MS.

Short and long term effect of catecholamines and various drugs upon adrenergic receptors on blood cells in vitro and in vivo have been thoroughly investigated. Reduced $\beta$-adrenergic receptor numbers have been reported during chronic $\beta$-adrenoceptor agonist treatment (Martinsson et al., 1987). Conversely, increased receptor sites on lymphocytes have been observed during long term administration of $\beta$-blockers (Van den Meiracker et al., 1989). During infusion of catecholamines the findings support an early up-regulation (Brodde et al., 1988; Tohmeh et al., 1980), and a later down-regulation (Larsson et al., 1989) of $\beta_2$-adrenoceptors on PBMCs. Exercise can increase the number and function of $\beta_2$-adrenoceptors on lymphocytes (Brodde et al., 1984, Butler et al., 1983). These findings have been attributed to sympatho-adrenal activation. However, an acute alteration in the number and affinity of $\beta$-adrenoceptors on PBMC in normal subjects after pharmacologically induced central sympathetic inactivation by clonidine has been reported (Zoukos et al., 1992b). These studies have further emphasized the important regulatory effect upon the number of $\beta$-adrenoceptors of the sympathetic nervous system output. Indeed in the human model of chronic sympathetic
denervation, as in patients with AF, an increase in the number of \( \beta \)-adrenoceptors has been reported (Bannister et al., 1981). Similarly, in MS patients, up-regulated \( \beta \)-adrenoceptors may reflect the previously described central autonomic dysfunction, occurring in the majority of these patients.

In an attempt to clarify the contribution of autonomic dysfunction in \( \beta \)-adrenoceptor up-regulation in MS, normal subjects (NS), secondary progressive MS and autonomic failure patients have been investigated. Studies were performed in the basal state and after reduction of central sympathetic outflow induced by clonidine.

4.4.1. Methods and subjects.

The previously reported MS, MSA, and PAF patients took part in this study. 15 normal subjects (7 males and 8 females, mean age 57.8±11; range 23 to 72 years) were studied as controls. Studies were carried out after clonidine as previously described. \( \beta \)-adrenoceptor densities and affinity were determined using a radioligand \(^{125}\) Iodocyanopindolol and intracellular CAMP production basal and after stimulation by Isoproterenol was measured using a commercial cyclic AMP \(^{125}\) assay kit (see Chapter 2).
4.4.2. The effects of clonidine on \( \beta \)-adrenoceptor density of PBMC in vitro.

PBMCs were washed in basal medium EAGLE (Gibco) under sterile conditions. The cells were cultured in Dutch modified RPMI-1640 (Gibco) supplemented with 10% foetal calf serum (FCS), 100 IU/ml penicillin, 10 \( \mu \)g/ml streptomycin, in the presence and absence of 4 \( \mu \)M clonidine; 4 \( \mu \)M being the peak plasma concentration of the drug 90 min after oral administration of 300 \( \mu \)g (Dolley et al., 1976).

4.4.3. Statistical analysis.

Data are presented as mean±s.e.m. Statistical analysis was done with Student’s paired t test and Wilcoxon rank sum test. The level of significance was taken as \( p<0.005 \).

4.4.4. Results.

4.4.5. Effects of reduced central sympathetic outflow on PBMC \( \beta \)-adrenoceptor densities in normal subjects.

The major findings are presented in Table 4.7. After clonidine, a statistically significant fall in BP (mean, systolic, and diastolic) at 30, and 60 mins was observed. Mean plasma levels of NA fell by 50% in 13 out of 15
subjects but high levels in two individuals affected the s.e.m. and the fall did not reach significance. Levels of Ad fell significantly. Plasma levels of dopamine (DA) remained unchanged. Significant increase in densities of \( \beta \)-adrenoceptors on PBMCs was observed at 30 and 60 min. In seven subjects a time course assessment demonstrated that \( \beta \)-adrenoceptors returned to baseline values 2 hours after clonidine (Fig 4.14.). The receptor affinity also changed with a substantial decrease at 30 and 60 min. The receptor binding assays were saturable and competitive at basal state. The receptor affinity was expressed as \( K_d \) in pM and the receptor density with \( B_{max} \) expressed as sites/cell and normal range of 134±40. The binding versus free linear regression analysis diagrams are presented in Fig 4.15. No changes in basal and stimulated intracellular production of cAMP were observed on PBMC at 0, 30 and 60 min after clonidine (Fig 4.16.). In vitro culture of PBMCs from four normal subjects in the presence of 4\( \mu \)M clonidine had no effect upon \( \beta \)-adrenoceptor density (Fig 4.17.). The increased \( \beta \)-adrenoceptor density at 30 and 60 min after clonidine was associated with a decrease in receptor affinity which did not permit saturability within the range of concentrations used 10-300 pM of \(^{125}\)Iodocyanopindolol (ICYP). To overcome this we repeated in 7 normal subjects the binding assays using a wider concentration range of ligand (10-500 pM). In Fig 4.18. are demonstrated the binding curves. Before clonidine, concentrations of ligand above 300 pM gave non-saturable,
Table 4.7. Changes in mean arterial blood pressure (MABP), systolic blood pressure (SBP), diastolic blood pressure (DBP), $\beta$-adrenoceptor density on PBMC and their affinity (kd) and plasma levels of noradrenaline (NA), adrenaline (Ad), dopamine (DA) and growth hormone before and after clonidine in normal subjects.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEAN ARTERIAL BLOOD PRESSURE (mm Hg)</td>
<td>90.9±2</td>
<td>80.17±2**</td>
<td>77.29±2**</td>
<td>75.86±2***</td>
<td>76.44±2***</td>
</tr>
<tr>
<td>SYSTOLIC BLOOD PRESSURE (mm Hg)</td>
<td>125.6±3</td>
<td>109.9±2***</td>
<td>109.9±2***</td>
<td>105.7±2***</td>
<td>102.7±3***</td>
</tr>
<tr>
<td>DIASTOLIC BLOOD PRESSURE (mm Hg)</td>
<td>73.3±2</td>
<td>65.4±2**</td>
<td>63.13±2***</td>
<td>62.47±2***</td>
<td>62.73±2***</td>
</tr>
<tr>
<td>$\beta$-ADRENOCEPTOR DENSITY ON PBMC (sites/cell)</td>
<td>1344±40</td>
<td>2831±459*</td>
<td>2114±316*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFFINITY ($K_D$ in pM)</td>
<td>126±17</td>
<td>250±47*</td>
<td>250±50*</td>
<td>250±50*</td>
<td></td>
</tr>
<tr>
<td>NORADRENALINE (pg/ml)</td>
<td>486±7.2</td>
<td>365±118</td>
<td>339±108</td>
<td>298±89</td>
<td>382±118</td>
</tr>
<tr>
<td>ADRENALINE (pg/ml)</td>
<td>45.7±7.2</td>
<td>38.1±7.7*</td>
<td>27.8±7*</td>
<td>27.5±4*</td>
<td>30.4±4.1</td>
</tr>
<tr>
<td>GROWTH HORMONE (mU/l)</td>
<td>1.90±0.84</td>
<td>1.53±0.34</td>
<td>6.24±1*</td>
<td>11.4±3.4**</td>
<td>12.1±3***</td>
</tr>
<tr>
<td>DOPAMINE (pg/ml)</td>
<td>45.1±9.4</td>
<td>43.9±8.2</td>
<td>48.2±8.5</td>
<td>46.7±14</td>
<td>56.5±19</td>
</tr>
</tbody>
</table>

***p<0.01, **p<0.02, *p<0.05 t-student paired test.
Fig 4.14. Time course changes in β-adrenoceptors after clonidine.

Time course changes in β-adrenoceptor density of PBMCs in seven normal subjects before and after clonidine. There is an increase in density at 30 and 60 minutes after clonidine with a return to baseline values at 120 minutes. (Points; mean and bars; s.e.m).
Fig 4.15. Scatchard plot analysis before and after clonidine.

Scatchard plot regression analysis of bound versus free (B/F) ligand. (1) represents a linear regression analysis of binding with normal range density and affinity receptors (before clonidine). (2) represents a linear regression analysis of binding with high density and low affinity of receptors (after clonidine at 30 and 60 min).
Fig 4.16. Intracellular cAMP before and after clonidine.

Basal and $10^{-4}$M isoproterenol-stimulated intracellular cAMP levels (mean±SEM) in peripheral blood mononuclear cells of five normal controls before (at rest) and at 30 and 60 minutes after clonidine. In each set there is an increase in intracellular cAMP levels after stimulation by isoproterenol (at rest from 1,060±120 to 2,220±600 fmol/10^6 cells, and after clonidine at 30 minutes from 1,030±300 to 2,670±720 and at 60 minutes from 1,120±60 to 2500±120 fmol/10^6 cells). However, there is no difference between either basal or stimulated levels when comparison is made before and 30 and 60 minutes after clonidine (*p<0.05, paired student's t-test).
Fig. 4.17. PBMC $\beta$-adrenoceptors after stimulation with clonidine.

Peripheral blood mononuclear cells from four normal subjects which were incubated in the presence and absence of 4$\mu$M clonidine for 2 hours. There was no difference in the $\beta$-adrenoceptor density in the presence (hatched histogram) or absence (empty histogram) of clonidine.
**Fig 4.18.** $^{125}\text{I} \text{CYP} \text{ binding before and after clonidine.}$

Percentage of specifically bound $^{125}\text{I} \text{CYP}$ over a range of concentrations from 20 to 500 pM. Curve 1 represented the binding before clonidine and curve 2 the binding after clonidine. Note that in curve 1, above 250 pM $^{125}\text{I} \text{CYP}$ the binding became nonspecific. This did not occur in curve 2 at 30 and 60 minutes after clonidine, indicating saturable binding of low-affinity receptors. (Each point represents the mean of three measurements. The intra-assay variation was less than 5%).
Fig 4.19. NA levels and β-adrenoceptors in NS, MSA, PAF and MS.

Basal levels of plasma noradrenaline (NA) (right panel) and β-adrenoceptor densities on peripheral blood mononuclear cells (PBMCs) (left panel) (mean ±SEM) in normal subjects (NS) and MS, MSA and PAF patients. Decreased plasma levels of NA were observed in PAF patients (90±37 pg/ml) and increased density of β-adrenoceptor was observed in MS and PAF patients (1890±204 and 2430±265 sites/cell respectively). *p<0.05 compared to NS (Wilcoxon rank sum test). Note the inverse correlation between NA plasma levels and β-adrenoceptor densities in PAF patients. The graphic presentation is in means±s.e.m., despite the use of non-parametric statistical analysis test.
Fig 4.20. Intracellular cAMP in NS, MS and PAF.

Basal and 10^{-4}M Isoproterenol stimulated cAMP (means±s.e.m.) production in peripheral blood mononuclear cells (PBMCs) from 5 normal subjects (NS), 5 MS patients and 5 PAF patients. Basal levels of intracellular cAMP are in NS 1020±54, in MS 1120±30 and in PAF 1100±60 fmol/10^6 cells. After β-adrenoceptor stimulation by isoproterenol intracellular levels of cAMP are in NS 1750±70, in MS 2900±100 and in PAF 3100±135 fmol/10^6 cells. *p<0.05 compared to the NS basal levels; +p<0.05 compared to the basal levels of MS and PAF and normal subjects stimulated levels (paired t-Student's test).
Table 4.8. β-adrenoceptor densities on PBMCs before and after clonidine in normal subjects (NS), MSA, PAF and MS patients.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-adrenoceptors sites/cell</td>
<td>NS</td>
<td>MSA</td>
<td>PAF</td>
</tr>
<tr>
<td>affinity (Kd, pM)</td>
<td>120±13</td>
<td>110±18</td>
<td>150±15</td>
</tr>
<tr>
<td>β-adrenoceptors sites/cells</td>
<td>243±265</td>
<td>218±327</td>
<td>214±463</td>
</tr>
</tbody>
</table>

*p<0.05 paired t-Student’s test.
non-specific binding results in accordance with previous observations (O’Hara et al., 1984). However, at 30 and 60 min after clonidine, even above 300 pM, the binding was saturable demonstrating detection of lower affinity receptors.

4.4.6. PBMCs β-adrenoceptors before and after reduction of central sympathetic outflow in MSA, PAF, MS, and NS. 

Basal state (before clonidine; time 0) β-adrenoceptor densities on PBMCs were within the normal range (NS; 1234±182 sites/cell) in MSA patients (1301±171 sites/cell) but elevated in MS and PAF patients (1890±204 and 2431±265 sites/cell respectively) Fig 4.19. Isoproterenol-stimulated intracellular production of cAMP indicated a functional β-adrenoceptor up-regulation (Fig 4.20). After clonidine, a significant increase in β-adrenoceptors on PBMCs was observed only in NS at 30 and 60 min (Table 4.8.).

4.4.7. Discussion. 

A high density of β-adrenoceptors on PBMCs has been demonstrated in secondary progressive MS and in PAF, while in MSA densities were similar to normal subjects. β-adrenoceptor up-regulation was functional in both MS and PAF patients, as increased intracellular cAMP was produced after β-adrenoceptor stimulation. In MS patients there
were no symptoms or signs of autonomic dysfunction affecting the cardiovascular system, as there were no symptoms or signs of postural hypotension. Furthermore their plasma levels of NA and Ad were within the normal range. In PAF however, with marked features of autonomic dysfunction there were low plasma levels of both NA and Ad. Thus they differed from MSA patients who, despite the clinical signs of autonomic failure, had normal levels of \(\beta\)-adrenoceptor density on PBMCs, and plasma levels of NA and Ad. The results in the two groups with autonomic failure suggest that basal levels of \(\beta\)-adrenoceptors are influenced by the levels of circulating catecholamines, as \(\beta\)-adrenoceptor up-regulation is associated with low levels in PAF patients and normal \(\beta\)-adrenoceptor density with normal levels in MSA patients. However, in the MS patients there was no inverse correlation between receptor number and neurotransmitter levels (Fig 4.19.), which differentiates them from the two groups with autonomic failure.

Short term up-regulation of \(\beta\)-adrenoceptors after clonidine, was observed only in NS. This was associated with a reduction of their affinity, indicating that \(\beta\)-adrenoceptors alter their coupling capacity with the catalytic component after sympathetic inactivation. As \(\beta\)-adrenoceptors either reside at the membrane surface, or may be internalized (Chuang et al., 1979), a rapid externalization/activation of such receptors with
different properties after reduction of sympathetic outflow could result in this observation. However, both ICYP and propranolol are lipophilic and therefore they would appear to be capable of crossing the membranes and identifying locations within the cells. Whether or not these newly appearing receptors reside at the membrane surface could be further clarified by using a hydrophilic antagonist such as CGP12177 for non-specific binding. Sympathetic activation leads to an alteration in the circulating lymphocytic subpopulations (Maisel et al., 1990). Since lymphocytic subpopulations have different \( \beta \)-adrenoceptor densities, such alterations may account for changes in \( \beta \)-adrenoceptor densities. However, probable changes in circulating lymphocyte subpopulations after sympathetic inactivation remain to be determined. Whether after sympathetic inactivation a more complex cascade of events involving changes in neuropeptides or hormones such as GH additionally contributes, remains unclear. The complexity of the acute adaptations of \( \beta \)-adrenoceptors after central sympathetic inactivation was further supported by the observations in the three groups of patients who demonstrate different forms of autonomic dysfunction. Nevertheless, the lack of response in all groups of patients, indicated that \( \beta \)-adrenoceptor adaptation process is highly sensitive and entirely relies upon intact sympathetic function (Zoukos et al., 1993). Thus, in MSA and MS patients, who demonstrated central sympathetic defects with abnormal GH responses to
clonidine but had a normal response in relation to BP and NA changes after clonidine, there was no change of β-adrenoceptors after clonidine.

4.4.8 Conclusions.

The comprehensive physiological assessment of autonomic function in MS patients including the basal state and responses to clonidine in MS, MSA, and PAF patients have helped to clarify a possible relationship between up-regulation of β-adrenoceptors and autonomic dysfunction in MS. In MS, the high basal density of β-adrenoceptor on PBMCs is unlikely to be the result of autonomic dysfunction, as plasma levels of NA and Ad are normal, compared to PAF in which basal β-adrenoceptors were high but levels of NA and Ad were low. Autonomic defects in MS, based on abnormal pressor responses in physiological assessment of autonomic function (Thomaides et al., 1993b) and haemodynamic and neurohormonal responses after clonidine, favoured a central autonomic involvement, as seen in MSA patients. Therefore, it is unlikely that autonomic dysfunction in MS could be a contributing factor in β-adrenoceptor up-regulation, as MSA patients, with a greater degree of central autonomic system dysfunction, had normal β-adrenoceptor densities. These observations indicate that increased expression of β-adrenoceptors in MS is independent of autonomic function and more likely is linked to immunological parameters characteristic of the inflammatory process.
CHAPTER 5

DISCUSSION

Since the late 1800s when MS was first described, the progress in clarifying its aetiology and successfully treating this disorder has been very slow and frustrating. Although the aetiology of MS is as yet not completely resolved, there are indications that genetic and environmental factors play a major role.

MS is an inflammatory disorder of the CNS characterized by breakdown of the BBB, traffic of activated immune cells into the CNS white matter, perivascular cuffing and demyelination. It is not yet clear whether the immune reaction is strictly directed against myelin sheath components. Furthermore, it is not known whether the immune reaction primarily starts in the CNS and is then followed by a secondary systemic phase which involves recruitment of activated lymphocytes from the periphery or is mainly systemic. A major part of research in MS therefore justifiably is focused on the genetic and immunological aspects of the disease. However, the urgent need for effective management of the disease, has diverted our attention from the causes of MS to the inflammatory process itself.

The purpose of this study was to investigate possible recovery mechanisms in MS. Alterations in \( \beta \)-adrenoceptors and IL-2Rs have been found to be closely linked with MRI and
disease activity. Although, β-adrenoceptor up-regulation on PBMCs was not a specific finding for MS and appeared to be linked with inflammatory processes in general (eg. rheumatoid arthritis), its importance was further emphasised by its association with IL-2R expression which implies activation of lymphocytes.

Three significant conclusions can be drawn from these findings. One is linked to the possible role of β-adrenoceptor expression in homeostasis during immune activation of lymphocytes, the potential of which could be therapeutically exploited. While pharmacological attention, so far, has been focused on preventing activation of T lymphocytes (with drugs such as cyclosporin), the presented in vitro studies show that functional down-regulation of IL-2Rs can be mediated through β-adrenoceptor stimulation. The other, confirms the validity of MRI in monitoring disease activity in MS and the third strongly supports the systemic nature of MS. These findings were further supported by a study in progressive MS patients where both increased disability and disease activity (monitored by clinical and MRI criteria) were associated with high densities of β-adrenoceptors and IL-2Rs on PBMCs. (unpublished data).

A major part of this thesis deals with the autonomic function in progressive MS. Although β-adrenoceptor expression was not found to be linked to autonomic dysfunction in progressive MS, both the physiological and
neurohormonal responses were able to detect minor abnormalities of central autonomic function. The contribution of hypothalamic dysfunction to the abnormal neurohormonal responses after clonidine, may indicate a wider dysfunction in the HPA in progressive MS which can influence the final outcome of the disease.

In future studies the significance of recovery mechanisms involving neural, endocrine and immune systems at the level of circulating lymphocytes could be linked with the diversity of clinical presentation in MS. MRI studies have been helpful in clarifying aspects of the natural history of MS and in monitoring disease activity. Undoubtedly, in benign MS patients, MS lesions appear less active. Therefore, a direct approach for future research would be to study possible recovery mechanisms in those patients. The emerging role for various immune mediators such as cytokines, promoting or inhibiting the inflammatory process, may also open new avenues for therapeutic intervention in MS and the recent results of the IFN-β therapy in MS further support the validity of these studies. Moreover, the rapid expansion of our knowledge of steroid actions may modify their therapeutic applications in MS. The induction of apoptosis in immunocompetent cells by steroids may be of vital importance in self-antigenic recognition and aberrant immune functions, such as those observed in MS, may be effectively abolished by appropriate manipulations of endocrine factors.
REFERENCES


of hormones on development and function of lymphoid tissue. Immunology 17:303.


Neuroimmunol. 25:203.


Invest. 90:2555.


Dohlman HG, Thorner J, Caron MG et al. (1991). Model systems for the study of seven transmembrane-segment


Engel G, Hoyer D, Berthold R et al. (1981). (±)-125Iodocyanopindolol, a new ligand for β-adrenoceptor: Identification and quantitation of subclasses of β-

Feder D, Im MJ, Klein HW et al. (1986). Reconstitution of $\beta_1$-adrenoceptor-dependent adenylyl;ate cyclase from purified components. EMBO J. 7:1509.


Gallo P, Diccino MG, Tavolato B et al. (1991). A longitudinal study on IL-2, sIL-2Rs, IL-4 and INFγ in


Halper JP, Mann JJ, Weksler ME et al. (1984). Beta


Increased lymphocyte beta-adrenergic receptor density in progressive multiple sclerosis is specific for the CD8⁺, CD28 suppressor cells. Ann Neurol. 30:42.


Lampson LA and 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 tumour involvement. J. Immunol. 136:4054.


Lefkowitz RJ, Caron MG. (1988). Adrenergic receptors:


noradrenaline content of the spleen are early indications of immune reactivity in experimental allergic encephalomyelitis in Lewis rat. J. Neuroimmunol. 23:93.


**Nagy E, Berczi I, Wren GE et al. (1983).** Immunomodulation by bromocriptine. *Immunopharmacology.* 6:231


**O'Hara N, Brodde OE. (1984).** Identical binding properties of (+) and (-) ¹²⁵Iodocyanopindolol to β₂-


Peter JB, Doctor FN, Tourtelotte WW. (1991). Serum and CSF levels of IL-2, sIL-2Rs, TNFα and IL-1β in chronic progressive multiple sclerosis: Expected lack of clinical
utility. Neurology 41:121.


Thomaides T, Chaudhuri R, Maule S et al. (1992). Differential responses in superior mesenteric artery blood flow may explain the variant pressor responses to clonidine in two groups with sympathetic denervation.


Van Den Meiracker AH, Man In't Veld AJ, Boomsma F et


Williams LT, Janett L, Lefkowitz RJ. (1976). Adipose


Zoukos Y, Thomaides T, Chaudhuri KR et al. (1994). Expression of β-adrenoceptors on circulating mononuclear cells in hypertensives and normotensives before and after
reduction of central sympathetic outflow by clonidine.

Blood Pressure 3:172.


APPENDIX
$T_2$ weighted images from patient 5; Table 3.3 (1st month)
$T_1$ weighted images from patient 5; Table 3.3. (1st month)
$T_1$ weighted images from patient 5; Table 3.3. (2nd month)
$T_1$ weighted images from patient 5; Table 3.3. (3rd month)
$T_1$ weighted images from patient 5; Table 3.3. (4th month)
$T_1$ weighted images from patient 5; Table 3.3 (5th month)
$T_1$ weighted images from patient 5; Table 3.3 (6th month)
$T_2$ weighted images from patient 4; Table 3.4 (1st month)
$T_1$ weighted images from patient 4; Table 3.4 (1st month)
$T_1$ weighted images from patient 4; Table 3.4 (2nd month)
$T_1$ weighted images from patient 4; Table 3.4 (3rd month)
$T_1$ weighted images from patient 4; Table 3.4 (4th month)
$T_1$ weighted images from patient 4; Table 3.4 (5th month)
$T_1$ weighted images from patient 4; Table 3.4 (6th month)