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Is Apolipoprotein-E modulated in peripheral blood mononuclear cells by IFN-β and is this to related patient responsiveness in Multiple Sclerosis.

A Dissertation submitted to
Trinity College, Dublin
For the Degree of Doctor of Philosophy

by

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December 2000.

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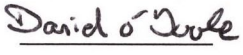
RESULTS

DECLARATION

I certify that none of the work in this thesis has been submitted for any degree or diploma at this, or any other, University and that all the work described in this thesis is entirely my own. I agree that Trinity College may lend or copy this thesis upon request.



Daniel O'Toole

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ABSTRACT

Multiple Sclerosis is a neurodegenerative disorder involving the attack of blood resident T-lymphocytes on the myelin sheath surrounding the neuron. This condition is particularly prevalent amongst white, northern European populations and appears to have both a genetic and environmental aspect to its incidence. Multiple Sclerosis commonly occurs in the Relapsing-Remitting manner, where periods of inflammatory attack are broken by longer phases of partial recovery. IFN-β has been shown to ameliorate attacks and improve recovery during remission. Apolipoprotein E is a protein found as part of the lipoprotein particles VLDL, HDL and chylomicron, where it binds to the ApoB/E receptor to deliver cholesterol and free fatty acids. These are much required by the regenerating neuron and the supportive oligodendrocyte to repair cell membranes and myelin sheaths. Physiological Apo-E is produced mainly in the liver, but both the brain resident astrocyte and the infiltrating macrophage also produce significant amounts. Control of its production is known to occur at transcriptional and post-transcriptional levels. Mutant Apo-E genotypes have been implicated in aberrant repair of damaged neurons in Alzheimer's disease and acute spinal injury. We have found that, in both the 1321N1 human and C6 rat astrocytoma cell lines studied, Apo-E mRNA transcription is inhibited by the inflammatory cytokines TNF-α and IL-1α, but not by IFN-γ. Unfortunately, no cellular or secreted Apo-E protein was detected from these cell lines. In both human primary monocytes and the monocyte cell line THP-1, Apo-E mRNA was inhibited by IFN-γ, and both translated and secreted Apo-E protein was inhibited. TNF-α, IL-1α and TGF-β stimulated Apo-E mRNA transcription, but did not affect cellular or secreted levles. IFN-β failed to alleviate the inhibition of Apo-E protein in these cells, and instead inhibited it in a similar manner to IFN-γ. No association was found between the Apo-E genotype and the production of Apo-E by primary monocytes.

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ABBREVIATIONS

Abs	Absorbance
Apo	Apoprotein/Apolipoprotein
bp	Base Pairs (of DNA)
BSA	Bovine Serum Albumin
cDNA	Complimentary DNA
CNS	Central Nervous System
CPM	Counts Per Minute
CSF	Cerebrospinal Fluid
CSPD	Disodium 3-(4-methoxyspiro(1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3,3.1.$1^{3.7}$]decan}-4-yl) phenyl phosphate
DIG	Digoxigenin
DMPC	Dimethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EDSS	Extended Disability Status Scale
EDTA	Ethylenediamine tetraacetic acid
FCS	Foetal Calf Serum
G6PDH	Glucose-6-Phosphate Dehydrogenase
HDL	High Density Lipoprotein
IDL	Intermediate Density Lipoprotein
IL-1	Interleukin-1
IFN	Interferon
kDa	kiloDaltons
LB	Luria Broth
LDH	Lactate Dehydrogenase
LDL	Low Density Lipoprotein
MOPS	3-[N-Morpholino]propanesulphonic acid
MRI	Magnetic Resonance Imaging
mRNA	messenger RNA
PAGE	Polyacrylamide Gel Electrophoresis
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PMA	Phorbol Myristic Acetate

PNS	Peripheral Nervous System
PPO	2,5,-Diphenyloxazole
RNA	Ribonucleic Acid
rpm	Revolutions Per Minute
RT-PCR	Reverse Transcriptase PCR
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
TBS	Tris Buffered Saline
TEMED	N,N,N,N,-Tetramethylethylenediamine
TGF-β	Tissue Growth Factor-β
TNF-α	Tumor Necrosis Factor-α
Tween 20	Polyethylenesorbitan monolaurate
VLDL	Very Low Density Lipoprotein
v/v	volume/volume
w/v	weight/volume

CHAPTER 1

INTRODUCTION

1.1 PHILOSOPHY OF THE STUDY.

This study was undertaken as an investigation into the influence of multiple sclerosis relapse phase inflammatory cytokines on apolipoprotein-E mRNA and protein levels, and also to investigate the modulation of this control by apolipoprotein-E genotype.

Multiple sclerosis (MS) is a neurodegenerative disease which until recently has had no effective therapy. New approaches to combating this disease involve the administration of anti-inflammatory cytokines to replace the corticosteroids, often used in treatment, which have proved unsatisfactory both in terms of efficacy and side-effects [Lyons et al 1988]. The most widespread of these anti-inflammatory drugs is Interferon-β, which has been available to MS sufferers since 1994.

The method of action of IFN-β is currently ill-defined. It appears to alter the ratios of inflammatory to non-inflammatory T-lymphocytes [Cherchi et al 1990], as well as regulating T-cell migration and infiltration into the brain seen during an MS relapse [Dhib-Jalbut 1997]. It has been proven in clinical trials to reduce incidence and severity of relapses, and also to slow the disease course, the first drug shown to do so [Jacobs et al 1996].

Apolipoprotein-E is the major lipid carrier in the brain [Chiba 1996], and as such is vital to neuronal repair of the cell membrane and myelin sheath. Genotypic variation in the Apo-E gene is shown to affect the ability of the brain to self repair, with the ε4 isoform leading to the early onset of Alzheimer's disease [Roses 1996] and failure to recover from acute brain damage [Samatovicz 2000]. Apolipoprotein-E has also been shown to be present in elevated levels in the cerebrospinal fluid (CSF) of MS patients in remission phase [Rifai et al 1987].

The inflammatory cytokines, which IFN-β is known to counteract in some circumstances [Satoh et al 1996], include IFN-γ. This cytokine has been shown to be elevated during MS relapse [Philippé et al 1996], and also to inhibit the production of

Apo-E by the monocyte [Brand et al 1993]. The monocyte is an infiltrating leukocyte, found in the brain of the MS sufferer [Raine et al 1990]. It can scavenge neuronal breakdown products and repackage them as HDL particles for delivery to the regenerating neuron [Posse et al 2000]. Other inflammatory cytokines are found in elevated levels during the relapse phase, some of which may also have a bearing on Apo-E production [Philippé et al 1996].

Recent reports indicate there may be a link between Apo-E genotype and the rate of progress of multiple sclerosis, with, as mentioned earlier, the ε4 allele being implicated [Gaillard et al 1998]. No data is currently available for the relationship between Apo-E genotype and the ability of patients to recover from MS relapses when under a regimen of IFN-β therapy.

1.2 APPROACH TO THE STUDY.

The specific objectives of the study were to measure quantitatively the amount of Apo-E produced in the astrocyte and the monocyte under the inflammatory conditions found during the MS relapse, and to attempt to examine the effects of IFN-β on this model. Apo-E was measured at the transcriptional level using Digoxygenin labelled cDNA probes where possible, and using RT-PCR where mRNA levels were prohibitively low for this technique. Translated protein and secreted protein were measured quantitatively by Western blot and scanning densitometry.

Where necessary, for greater sensitivity, ^{35}S labelling of cellular protein was used, followed by immunoprecipitation of Apo-E and visualisation in SDS-PAGE using PPO scintillant.

A model was set up originally using the astrocyte cell lines (1321N1 human and C6 rat), followed by a monocyte cell line (THP-1) and finally using primary monocytes prepared from freshly collected donor buffy coats. These buffy coats were then genotyped using RFLP, and the prevalence of the ε4 allele noted in comparison to

Apo-E production. Apo-E protein levels in the plasma derived from these buffy coats were also noted.

Since Apo-E cross-reactivity has been noted with FCS lipoproteins, all experiments had to be performed in FCS free conditions. This may have a bearing on cell survival over the time-course of an experiment, so LDH assays were used in all cases to test for cytotoxicity of cells under such stress.

1.3 MULTIPLE SCLEROSIS.

1.3.1 PATHOGENESIS.

Multiple sclerosis, first described by Jean-Martin Charcot in 1868, is a chronic progressive disease involving the inflammatory demyelination of neurons in the central nervous system [Colover 1988]. This leads to the formation of lesions, or "sclerotic plaques", where astrocytes have died and formed scar tissue. MS plaques frequent around ventricles, gray-white matter junction, optic nerves, brainstem and spinal cord [Lucchinetti et al 1996]. Optic neuritis, or "white flashes", when the eyes are closed can be an early indicator of disease [Kahana et al 1976].

Early lesions are typified by myelin disintegration due to microglial and macrophage reaction to neurons. Damage to the blood brain barrier, which normally preserves the brain's immunoprivileged status, leads to perivascular lymphocytic and plasma cell infiltration, causing oligodendroglial loss. Later lesions show total loss of myelin and oligodendroglia, disappearance of perivascular inflammation, marked astrogliosis, and some degree of axonal loss [Silber and Sharief 1999].

Four clinical categories of MS have been described [McDonnell and Hawkins 1996]:

1. Relapsing-remitting (RR) MS (30%): Clearly defined relapses with full recovery or with sequelae and residual deficit upon recovery; the periods between disease relapses are characterized by a lack of disease progression.
2. Primary-progressive (PP) MS (10%): Disease progression from onset with occasional plateaus and temporary minor improvements.
3. Secondary-progressive (SP) MS: Initial RR disease course followed by progression with or without occasional relapses, minor remissions, and plateaus.
4. Progressive-relapsing (PR) MS: Progressive disease from onset, with clear acute relapses, with or without full recovery; the periods between relapses are characterized by continuing progression.

Clinical diagnosis of definite MS is achieved through a combination of relapse identification and MRI scanning, and through the presence of oligoclonal bands in Cerebro Spinal Fluid (CSF) samples [Giles and Wroe 1990]. There are also two severity outcomes described [Flachenecker and Hartung 1996]:

1. Benign MS: Disease in which the patient remains fully functional in all neurological systems 15 years after disease onset.
2. Malignant MS: Disease with a rapid progressive course, leading to significant disability in multiple neurological systems or death in a relatively short time after disease onset.

The course of relapse/remission is normally assessed using the Extended Disability Status Scale (EDSS) [Ravnberg et al 1997, etc], in which patient disability is estimated by a physician using a points scale for loss of function, ability to perform certain tasks. Zero on the scale represents no disability, ten represents death of the patient. There are a number of variations on this scale, such as the Functionality Scale and the MS Disability Scale [Amato and Ponziani 1999].

Gadolinium enhanced magnetic resonance imaging is used to give an objective quantification of severity of attack [Giugni et al 1997], and proton emission tomography and nuclear magnetic resonance imaging are also useful in visualising the sclerotic plaques. However, the correlation between EDSS progression and MRI lesion load, although a useful guide, is not always complete, as certain plaques are located in more sensitive areas of the brain [Dastidar et al 1999].

1.3.2 EPIDEMIOLOGY.

There is still much debate as to whether predisposition to MS is primarily genetic or environmental. There are rare recorded cases of MS "epidemics", such as in the Faroe Islands during World War Two, where the arrival of a non-native population of US servicemen coincided with the arrival of the previously unknown disease [Kurtzke 1987]. But in general, as one approaches the poles, incidence increases [Esparza at al 1995]. It is particularly prevalent amongst northern European populations [Mirsattari SM et al 2001], but it is not unknown elsewhere. Generally, the racial prevalence is whites > blacks > Asians [Alter and Harshe 1975].

Children of northern European ancestry who are moved to warmer climates, such as South Africa, Australia or Israel [see Table 1.1], before the age of 7 years, appear to have the same likelihood of becoming sufferers as those born locally [Weinshenker 1996]. It must also be noted, in this well-documented Israeli study, that most migrants were of Jewish stock. This would suggest a viral source, to "prime" the inflammatory response. Such diseases may be endemic in the local population or heritable familialy.

Table 1.1: MS incidence in native and migrant populations.

MS PREVALENCE IN VARIOUS POPULATIONS. (per 100,000)		MS PREVALENCE IN NON-NATIONALS IN ISRAEL. (per 100,000)	
Oslo	80	Germany	51
Denmark	64	Poland	30
USA (Northern States)	64	Holland	30
Northern Scotland	57	Great Britain	30
Northern Ireland	51	Yugoslavia	11
Sweden	51	Bulgaria	9
Estonia	51	Turkey	7
Baltic Republics	35	Iraq	7
Moscow	33	Iran	7
Belorussia	28	Egypt	6
Switzerland	23	Morocco	6
Kiev	23	Tunisia	6
Cherkassy region	23	Libya	6
Roman	21	Algeria	6
Prikarpatsky region	20	>>Israeli natives<<	4
Zakarpatsky region	16	Yemen	3
		Aden	3

[Alter et al 1962]

This model may function through neuronal expression of viral proteins that have cross reactivity with myelin sheath proteins [Ota et al 1990]. T-lymphocytes and other cells may then begin to launch an immune response against all myelin containing cells. This is termed the "Molecular Mimicry Model". Northern Europeans could be more exposed or susceptible to any such causative viruses. This could also explain why the mean age of onset is around 30 years of age, as many childhood viruses, such as shingles, take several years to migrate to the Central Nervous System (CNS) and be activated. Epstein-Barr [Haahr et al 1995] and cornavirus [Tabbot et al 1996] are amongst the viruses that have been investigated as priming agents, which is now readily done through PCR analysis of blood or CSF.

Viral persistence may also be a factor, causing Inducible Nitrous Oxide Synthase (iNOS), Tumour Necrosis Factor Alpha (TNF-α) and Interleukin-6 (IL-6) expression in astrocytes, triggering inflammatory attack and disrupting oligodendrocyte function [Buchmeier and Lane 1999].

Mutant TNF-α alleles, a host of mutant myelin proteins [Sandberg-Wolheim et al 1995] and mutant mitochondrial DNA [Kalman et al 1995, 6] in Caucasians have also been implicated. Another genetic factor to be considered is the gender distribution, in which females are 50% more likely to contract the disease [Warren and Warren 1996]. Familial aggregation in this disease is not strong, but the risk to a first-degree relative of a patient with multiple sclerosis is at least 15 times that for a member of the general population [McAlpine 1965]. Increased risk for daughters of female sufferers was particularly noted. A survey of 10 multiple sclerosis clinics across Canada found 27 monozygotic and 43 dizygotic twin pairs with multiple sclerosis in at least 1 of each pair. Seven (25.9%) of the monozygotic pairs and 1 (2.3%) of the dizygotic pairs were concordant for multiple sclerosis [Ebers et al 1986]. The concordance rate for non-twin siblings was 1.9%. The higher concordance rate in monozygotic twins despite the low recurrence risk in families is consistent with a polygenic model.

1.3.3 BIOCHEMISTRY.

The mode of action of MS is the attack on the myelin layer, and it's subsequent stripping, by the T-lymphocyte and the macrophage. These cells are normally involved in the phagocytosis of cancerous or virally infected cells, or those undergoing apoptosis. This is typically mediated by expression of Cellular Adhesion Molecules (CAMs), such as V-CAM, I-CAM and E-selectin [McDonnell et al 1999], and a number of chemotactic factors, such as IL-8 [Brosnan and Raine 1996], by the compromised cell. There is also a related incidence in the cerebrospinal fluid of the soluble breakdown products of these molecules: sV-CAM, sI-CAM and sE-selectin [Hartung et al 1995]. The elevated levels of these proteins may become a useful diagnostic tool in assessing MS relapses quantitatively.

This combination of CAMs and chemotactic factors allow lymphocytes and macrophages to migrate from the blood deep into tissues, where they adhere and begin to secrete toxic substances, such as oxygen free radicals [Smith et al 1998], nitric oxide [Giovannoni et al 1998], and leukotrienes [Shamsuddin et al 1992]. These chemicals are known to be more toxic to neurons than to astrocytes [Sochocka et al 1994], hence astrocytes can persist at sites of lesion formation.

The expression of the chemotactic/adhesion molecules is known to be modulated by cytokines and steroids, and elevated levels of integrins such as Lymphocyte Function-associated Antigen 1 (LFA-1, binding I-CAM) and Very Late Antigen 4 (VLA-4, binding V-CAM) are similarly seen on the T-lymphocyte [Luján et al 1998]. Generally, pro-inflammatory cytokines (IFN-γ, IL-1α, TNF-α) upregulate, and anti-inflammatory cytokines (IFN-α, IFN-β, TGF-β) downregulate them. These cytokines can be secreted by local neurons, astrocytes, or even the attacking lymphocytes and macrophages themselves.

Cytokine fluctuations also cause a shift in T-lymphocyte sub-populations during relapse, from more benign types to more aggressive TNF-α and IFN-γ producing

species [Pashov 1997]. This accelerates the inflammatory response. B-lymphocytes may also compound the inflammation once they have infiltrated into the CNS through production of antibodies against MBP or other neuronal surface proteins [Olsson 1990]. These antibodies can then target neurons for further destruction.

Recent evidence suggests that pro-inflammatory cytokines may actually also mediate remyelination and repair later in the relapse[Sharief 1998], depending on the temporal and spatial relation of cytokines to their targets. Therefore, certain cytokine therapies may not appear to be as beneficial as expected at certain phases of the relapsing-remitting cycle.

An animal model of MS is available called Experimental Allergic Encephalitis (EAE), which was first developed by Thomas Rivers in 1935 [Rivers and Schwentker 1935]. This technique ultimately led to the identification of MS as an autoimmune disease. It can be induced in any mammal by injection of homogenised brain and spinal matter from one animal onto the CNS of another. Demyelinating, MS-like, attacks begin to occur after several days, mimicking the relapsing-remitting model. Novel therapies that ameliorate EAE in animals generally have good efficiency when used in human MS trials.

1.3.4 THE MYELIN SHEATH.

Myelin itself is a coating of "insulating" material encasing neuronal axonal processes. It is found in both the CNS, where it is deposited by the oligodendrocyte, and the peripheral nervous system (PNS), where it is formed by the Schwann cell. These cells form processes around the axon, and deposit myelin in layers, particularly immediately after axonal growth, such as in the developing brain [Figure 1].

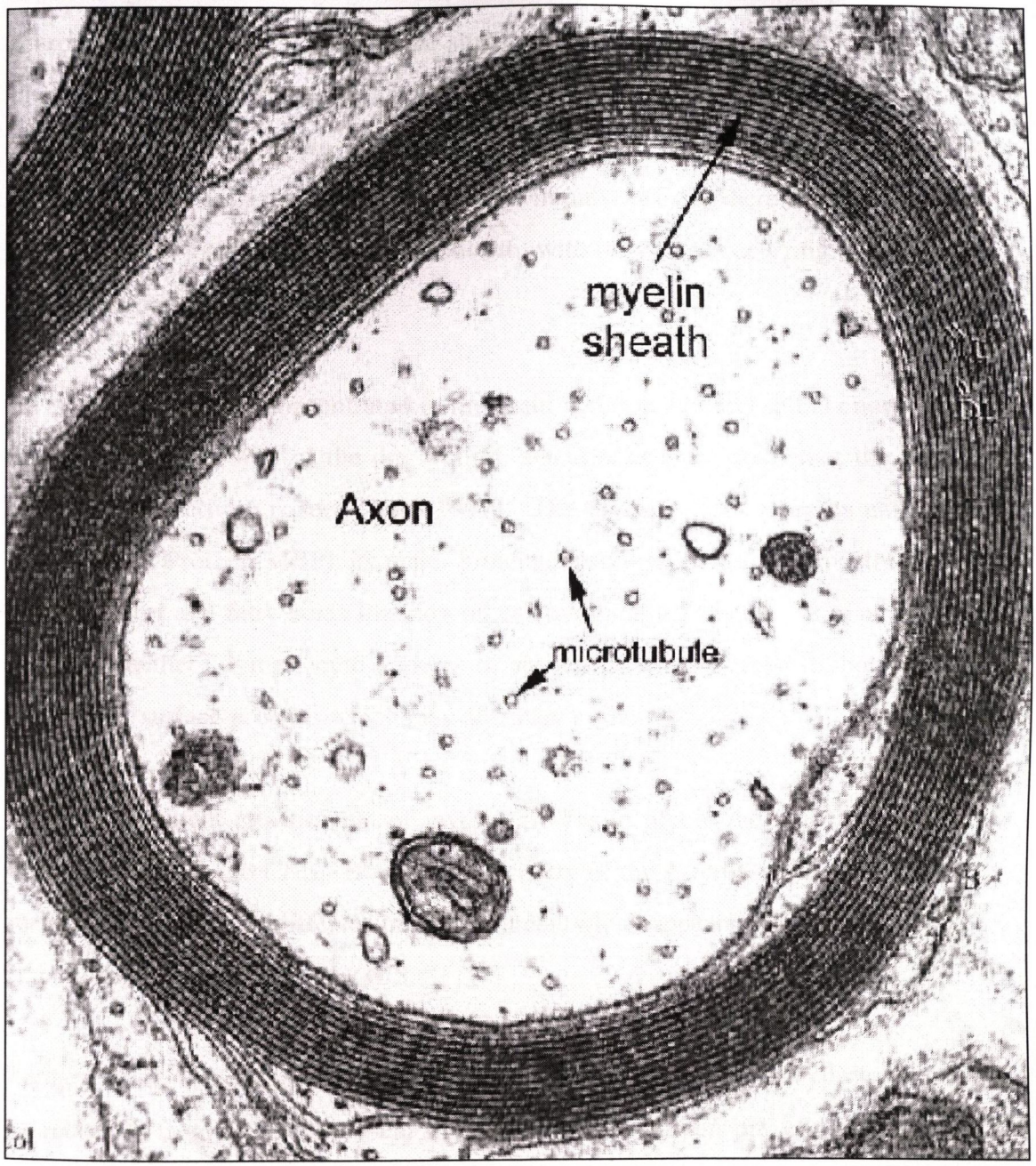

Figure 1.1: The myelin sheath: *Scanning micrograph of a rat spinal chord axon. The myelinating Schwann cell process can be seen at the lower right of the axon, just inside the myelin layer.*

The function of the myelin sheath is to enhance the depolarisation strength and speed in the firing neuron. Voltage gated ion channels, which propagate the signal along the neuronal membrane, lose ions to the surrounding fluid when myelin is not present. This can both dissipate the signal, and slow the depolarisation speed. It may also cause a delay in re-uptake of ions required for subsequent firings. It has been calculated that the maximal velocity of nerve impulse occurs where the ratio of naked neuron to total axonal diameter is about 0.6, with misfirings occurring at below this ratio [Waxman 1980].

Myelin is particularly concentrated in the brain white matter and spinal chord, where it accounts for 50-60% of the dry weight, and it is at these areas that the greatest inflammatory activity is seen [Ilyas 1997]. The myelin sheath takes its name from Myelin Basic Protein (MBP), its major protein constituent, although it mostly consists of cholesterol and fatty acids like any other biological membrane. It is against this protein that the T-lymphocyte appears to act during the MS relapse, but there are many other surface proteins which may also play a role.

The major protein constituents of myelin are found glycosylated in the PNS and unglycosylated in the CNS. Hence the reactivity of the T-lymphocyte to the myelin surface proteins can be different in the two areas, which appears to be the case as PNS neurons are seldom affected by MS.

Remyelinating oligodendrocytes, which are vital to the MS remission phase, can only be recruited from areas very local to the neuron, which can prevent full repair of larger lesions [Franlkin and Blakemore 1997]. Sclerotic plaques of greater than a few millimeters in diameter are generally never repaired fully. These cells are believed to be present in the adult brain at much lower levels than in the infant [Prineas et al 1989], which may be another reason why MS only becomes manifest in later life. The astrocytic scarring found in the MS lesion may also prevent migration of the oligodendrocytes to the areas requiring remyelination after a relapse [Fok-Seang et al 1995]. At the appropriate time, MBP is expressed via the transcription factors SCIP

and Gtx in oligodendrocytes, and repressed by NFI in non-myelinating cells [Kamholz 1996].

1.3.5 CURRENT AND PROSPECTIVE THERAPIES.

There are several therapies currently in use for MS, many of which have proved highly unsatisfactory. Muscle relaxants and painkillers are still, unfortunately, the mainstays of MS treatment. Global anti-inflammatory drugs, such as adenocorticotrophic hormone, methylprednisolone, azathioprine and mofetil have been administered for several years, and although these may ameliorate relapse severity, they can have severe side effects and do not actually slow the course of the disease. Attempts have been made to block the CD4 and CD18 receptors on the T-lymphocyte [Rumbach et al 1996] to prevent their activation, although this also has obvious general immunosuppressive consequences.

Others have attempted to flood the bloodstream with large amounts of MBP [Weiner et al 1993], in the hope that lymphocytes and macrophages will become 'tolerised' to these proteins, or else target the circulating drug in preference to native myelin protein. More recently, attempts have been made to block the action of TNF-α using neutralising antibodies [Hohlfeld 1996], and to specifically target lymphocyte sub-populations for destruction [Sipe et al 1996].

Experiments have continued with neurotrophic factors such as Tissue Growth Factor Beta (TGF-β), the physiological levels of which have been shown to correspond with inhibition of EAE [Ishikawa et al 1999], and Insulin-like Growth Factor 1 (IGF-1), affecting general brain cell populations. Other drugs are targeted to affect oligodendrocytes more specifically, using Glial Maturation Factor Beta (GMF-β) and heregulin [Marchionni et al 1993]. As with many other neurodegenerative diseases, neuronal and oligodendroglial progenitor cells are being tested for their ability to reintegrate into the brain and to differentiate into active cells [Lundberg et al 1996].

In the early 1990's, researchers noticed that IFN-γ appeared to exacerbate inflammatory activity in animals with EAE [Mustafa et al 1991]. The logical next step was to use an anti-inflammatory cytokine to counteract this effect. IFN-α was the first such drug to be tested and receive US Food and Drugs Administration (FDA) approval. Improved versions were soon produced, with better glycosylation patterns and efficacy, but although they were shown to lessen severity of relapse, disease course remained constant. Attention was soon turned to another drug, IFN-β.

1.4 IFN-β.

1.4.1 STRUCTURE.

IFN-β, in its native form, is a 165 residue glycosylated amino acid expressed in neurons, astrocytes, lymphocyte and macrophage, amongst other cell types. The interferons are biologically potent cytokines that can be divided functionally into two classes. Type I interferons are generally anti-inflammatory in nature, whilst type II are generally pro-inflammatory. Human type I interferons comprise a family of 13 IFN-α subtypes and a single species each of IFN-β and IFN-ω [Platanias et al 1996]

Computer modeling suggests that the globular structure of type I IFNs consists of a bundle of 5 α-helices, which might form two polypeptide domains [Kontsek 1994]. There are disulfide bonds between cysteine 29 and cysteine 139, which may stabilize the configuration. IFN-β, IFN-ω and some IFN-α subtypes are glycoproteins, but the sugar moiety does not appear to be critical to its binding to the type I IFN cellular receptor. Two conservative hydrophilic regions associated with the amino acids 30-41 and 120-145 appear to constitute the basic framework of receptor recognition site in type I IFNs. Heterogeneity in the segments amino acids 30-41, 120-145 and the variable hydrophilic regions 23-26, 68-85 and 112-121 are responsible for structural and functional individuality among human type I IFNs.

They have 20% overall sequence homology, which means they have almost identical secondary and tertiary folding. Fragments of the type I interferons do not appear to have any biological activity, suggesting almost all of the domains contribute somewhat to the binding site.

Only one receptor has been identified for this entire class of molecules, suggesting different binding affinities and redundancy in the family. This is termed the type I human interferon receptor (IFN-α/βR). It has a soluble analogue termed p40, which is a potent inhibitor of type I IFNs, and antibodies against p40 completely block the activity of type I IFNs in human cells. Two distinct regions have been cloned: a 1.5kb coding for a 51kDa transmembrane protein, IFNAR1, and a 4.5kb coding for p40, IFNAR2 [Novick et al 1995].

1.4.2 FUNCTION.

The interaction between IFN-β and its receptor seems to be a complex event which triggers simultaneously antiviral, antiproliferative and immunomodulatory actions. Interferon activity is species restricted, so pertinent pharmacological information is derived from studies in human cell culture and in humans. The binding of IFN-β to its receptor causes receptor phosphorylation at tyrosine residues through the cytoplasmic Janus tyrosine kinase enzymes JAK1 and Tyl2 [Lewerenz et al 1998]. These events induce the expression of a number of gene products, such as 2',5' oligodenylate synthase, protein kinase and indoleamine 2,3-dioxygenase that are believed to be the mediators of its biological activity [McLaurin et al 1995]. It has many functions, many of which are yet to be documented:

1: Regulation of T-cell and macrophage function, such as activation, proliferation, and suppresser cell function.

2: Modulation of production of other cytokines: Pro-inflammatory cytokines are suppressed and anti-inflammatory cytokines are stimulated.

3: Regulation of T-cell migration and infiltration into the CNS across the blood

brain barrier.

All of these abilities would appear to make IFN-β an ideal candidate for use as a treatment for multiple sclerosis.

1.4.3 THERAPY.

Clinical trials have been held in many countries since 1993 using IFN-α and IFN-β from a variety of sources, after low toxicity was shown in primary human neuronal cells [Rosenblum et al 1990]. Of the two, IFN-β has been found to be the drug of choice, because of its greater efficacy and its less severe side effects. The drug received FDA approval for prescription by physicians in the US 1993, and is now also available, on prescription, in Ireland and the UK for the treatment of both Relapsing-Remitting and Secondary Progressive MS.

The forms of IFN-β currently used are the recombinant bacterial form, IFN-β-1b ("Betaseron" by Berlex, containing a cysteine instead of a serine residue at amino acid 17), and the recombinant animal cell line form, IFN-β-1a ("Rebif" by Serono). Of the two, only IFN-β-1a is glycosylated, and hence is identical to naturally occurring IFN-β, but both drugs are of similar efficacy [Antonelli et al 1999]. They are both administered intravenously, but recent oral trials have also been promising [Nelson et al 1996].

IFN-β is most commonly administered by subcutaneous injection 3 times per week of between 2 and 12 millions units. This is normally as a saline resuspended lyophilised powder containing the recombinant protein and human serum albumin as a carrier and stabiliser. Preloaded syringes are also available, and the drug is quite routinely self-administered. The vast majority of the drug is lost before reaching the relevant site at which it is needed [Salmon et al 1996], due to the low stability of IFN-β and its clearance from the bloodstream by other body cells and by proteolytic cleavage.

1.4.4 BENEFITS.

Patients are normally accepted for the MS drugs trials only at similar ages, duration after diagnosis and level of disability [Whitaker et al 1995]. After 2 years of treatment with $12x10^6$ U of IFN-β-1b, a 50% reduction in relapse rate was observed in one US Relapsing-Remitting study [Berlex 1999]. There was a measurable delay in progression on the EDSS scale, and decreased lesion load in MRI scans [Table 1.2]. Other, double-blind, placebo controlled, studies have been conducted which corroborate this evidence in other populations. After these initial findings, longer term trials have been conducted with similar results. Also, testing has been successful for a trial group of Secondary Progressive MS sufferers [European Study Group 1998].

1.4.5 DISADVANTAGES.

Side effects often include flu like symptoms, fevers and chills, and some evidence of depression, but are otherwise minimal [European Study Group on IFN-β in MS 1998]. Injection site necrosis can also be a problem. Liver enzyme levels may become abnormal, but in all cases this resolves spontaneously.

Another problem to be aware of in recombinant IFN-β therapy is the existence of neutralising antibodies [Kivisakk et al 1997] that may affect the treatment and can arise within 6 months in up to 25% of all patients. These antibodies tend to decrease the therapeutic effect in terms of relapse rate, but not in terms of speed of progression on the EDSS scale.

Table 1.2: Benefits of IFN-β-1btherapy.

Dose/2days	0mg	0.05mg	0.25mg
Exacerbations per year	1.31	1.14	0.9
Exacerbation free patients after 2 years	16%	18%	25%
Mean days in relapse	44.1	33.2	19.5
Mean EDSS change	+0.21	+0.21	-0.07
Mean MRI lesions	+21.4%	+9.8%	-0.9%

Some of the quantifiable benefits seen in a two year double-blind, placebo controlled trial of IFN-β. [Berlex 1999]

Unfortunately, the current cost of therapy is upwards of £10,000 annually, leading to problems with funding for prescriptions, especially in developing countries. Recent studies indicate that when loss of earnings and other health care costs are considered, this may actually be, overall, a cost-saving treatment [Kendrick and Johnson 2000].

1.5 APOLIPOPROTEIN-E.

1.5.1 THE LIPOPROTEIN PARTICLE.

Lipids are a class of water insoluble organic molecules, which include fatty acids, triglycerides, phospholipids and cholesterol. In the human body, lipids are derived from fats following ingestion and intestinal absorption. They are also endogenously synthesised from carbohydrate and proteins. Phospholipids and cholesterol are important molecular components of cell membranes and cellular organelles.

Cholesterol is transported in plasma in spherical particles called lipoproteins. These contain an apolar core of triglyceride and cholesteryl ester surrounded by a surface coat of phospholipid, cholesterol and proteins. These proteins are the apolipoproteins, which target certain types of lipoprotein to certain tissues. Five main classes of lipoprotein particle exist in plasma: very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). The fifth, post-prandial, type is a triglyceride rich lipoprotein known as the chylomicrons. The proportion of cholesterol, triglyceride, phospholipid and protein vary between, and indeed within, each lipoprotein family [Table 1.3].

Two main pathways of lipoprotein metabolism exist [Figure 2]. In the first, exogenous pathway, chylomicrons are formed in the intestine and transport dietary triglyceride to tissues where it is removed by the action of the enzyme lipoprotein lipase. The resulting chylomicron remnants bind to the chylomicron remnant

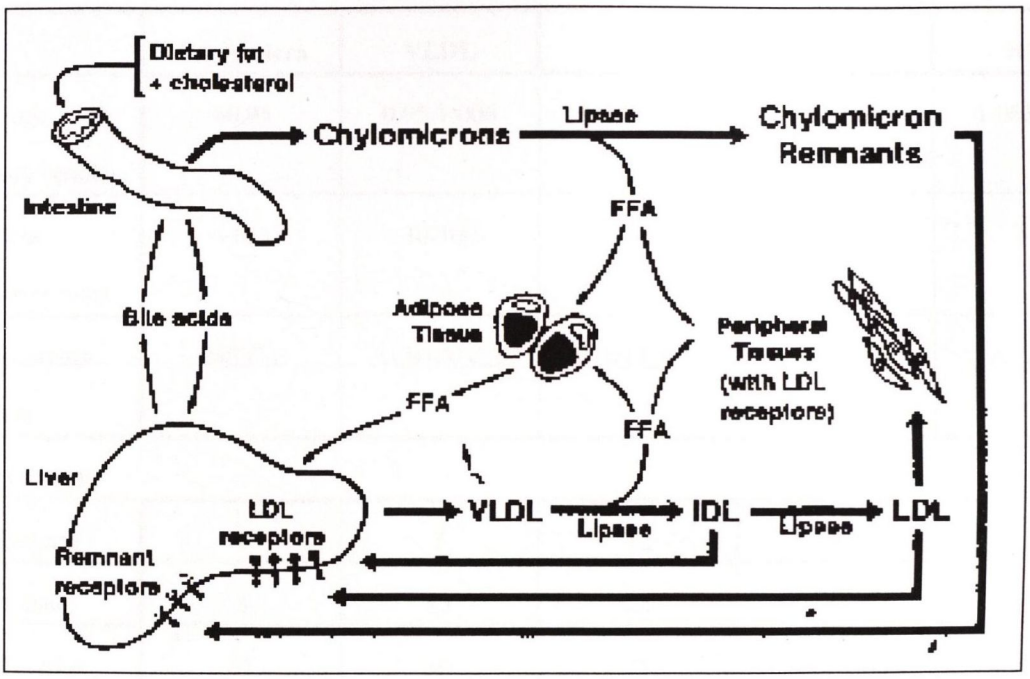

Figure 1.2: Overview of lipoprotein metabolism: *Diagram showing the endogenous and exogenous pathways involved in the distribution of cholesterol and lipid from the intestine to the liver, and from there to the peripheral tissues.* [Mahley 1988]

Table 1.3: Composition of the Lipoprotein Families.

	Chylomicra	VLDL	IDL	LDL	HDL
Average density (g/mL)	<0.95	0.95-1.006	1.006-1.013	1.013-1.063	1.063-1.21
Particle diameter (nm)	>100	30-100	30	19-25	7-10
Apoproteins present	B48,C,E	AI,B100,C,E	B100,E,B48	B100,C	AI,AII,E
% by weight					
Cholesterol	1	7	12	8	2
Chol. ester	3	13	22	37	15
Triglyceride	85	50	26	10	4
Phospholipid	9	20	22	20	24
Total protein	2	10	18	25	55

The physical parameters, apolipoprotein content and cholesterol/lipid composition of the various major lipoprotein classes. [Feher and Richmond 1995]

receptors on hepatic cells which recognise Apo-E and the chylomicron remnants are internalised and catabolised [Gardner and Mayes 1978]. In the second, endogenous pathway, VLDL particles are synthesised in the liver and transport endogenous triglyceride to other tissues, where they are also removed by the action of lipoprotein lipase. Cholesterol, phospholipid and apolipoproteins are transferred to HDL, resulting in the formation of VLDL remnants or IDL particles. Some IDL is removed by the liver via the B/E "scavenger" receptor, but most is converted to LDL by lipase. Whatever their destination, LDL particles are internalised and metabolised by lysosomal enzymes to release free cholesterol and triglyceride for the cell's use.

Secretory proteins such as Apo-B100 or Apo-E are synthesised on polysomes bound to the cytoplasmic surface or the rough endoplasmic reticulum. Lipid is synthesised in the smooth endoplasmic reticulum. The Microsomal Triglyceride Transfer Protein (MTP) is located in the lumen of these microsomes, where it is proposed to act in the assembly of lipoproteins by transferring lipid to nascent apolipoproteins as they enter the lumen of the endoplasmic reticulum [Gordon 1997].

1.5.2 APO-E STRUCTURE.

Apolipoprotein-E is a glycoprotein of molecular weight 34,145 Daltons with 299 residues which can be found in all mammals [Mahley 1988]. It is coded for by a 3.7kbp gene on chromosome 19 and consists of four exons and a TATAATT promoter sequence occurring approximately 30bp upstream from the transcription initiation site [Freitas et al 1998]. It is in close proximity on the chromosome to the Apo-CII locus, which has been implicated in MS susceptibility in one study [Zouali et al 1999]. The LDL receptor gene and the Apo-CI genes are also located nearby on this chromosome.

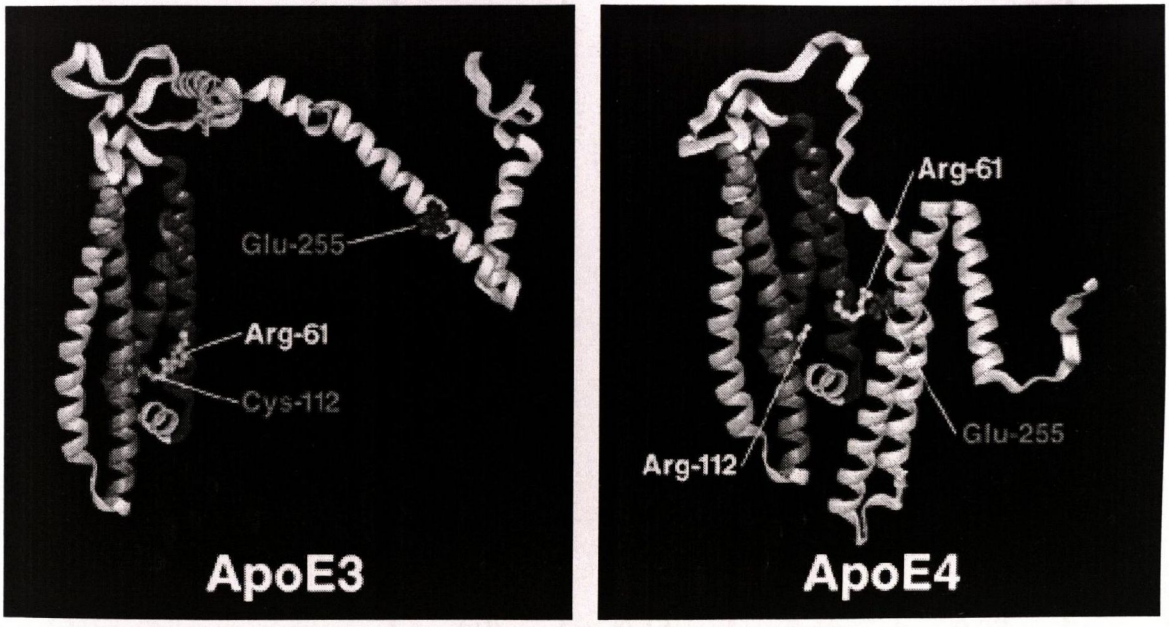

Figure 1.3: Apo-E ε3 and Apo-E ε4 Structure: *Altered tertiary structure of the Apo-E protein due to the interaction of arginine and glutamine residues in the α-helical subunits.* [Dong and Weisgraber 1996]

Table 1.4: Apo-E allele frequencies.

Population	Number	Allele Frequency		
		ε2	ε3	ε4
Irish	308	0.05	0.80	0.15
Americans	1209	0.07	0.79	0.14
Germans	1031	0.08	0.77	0.15
Finns	615	0.04	0.73	0.23
Dutch	200	0.08	0.75	0.17
Scottish	400	0.08	0.77	0.15
Nigerians	365	0.03	0.67	0.30
Sudanese	103	0.08	0.63	0.29
Japanese	319	0.08	0.85	0.07
Chinese	190	0.10	0.83	0.07
Indian	142	0.05	0.83	0.12
New Guineans	110	0.14	0.49	0.37
Australian Aborigines	64	0.00	0.74	0.26
Mayans	135	0.00	0.91	0.09

The incidence of the major Apo-E allelic forms in a variety of populations. [Smith 1996]

Crystallographical studies indicate the presence of two specific structural/functional domains: a phospholipid binding domain at the carboxy terminal and an LDL receptor (Apo-B/E receptor) binding domain at the amino terminal [Zaiou et al 2000]. It has 4 antiparallel α-helices at the latter terminus, and receptor binding activity has been localised to a basic residue rich charged region at residues 136-150 on α-helix 4. There are seven acidic cysteine rich repeat units at the LDL receptor binding site, which would appear to be complementary to this moiety. Calcium ions seem to be crucial to the affinity of Apo-E for the LDL receptor, and its absence may by used in experiments where re-uptake of Apo-E into the cell may influence protein secretion measurements.

1.5.3 APO-E FUNCTION.

Apo-E is found in chylomicra and VLDL and also in the HDLc, HDL_2, and β-VLDL lipoprotein remnant complexes. It exhibits specificity for the Apo-B/E receptor, which is expressed in liver, macrophage, astrocyte and oligodendrocyte cells, amongst others [Elshourbagy et al 1985]. Apo-E is normally found in human serum at a concentration of between $0.1gL^{-1}$ and $0.6gL^{-1}$, but is found in increased concentrations during hyperlipoproteinemia, pregnancy, choleostasis and during the repair and remodeling of damaged neurons [Brewer 1988].

Unlike the other apolipoproteins, which are synthesised primarily in the liver and small intestine, Apo-E is produced by a variety of organs and tissues, suggesting important additional physiological roles. In primates, the brain is the second largest site of Apo-E synthesis, expressing approximately one-third of the Apo-E found in the liver [Elshourbagy et al 1985]. Astrocytes are the major cell type responsible for Apo-E synthesis in the brain [Pitas 1987], whereas the neuron produces none, but does express the LDL receptor. It can also be located in cerebrospinal fluid, probably from the astrocytic or other glial source [Guyton et al 1998]. In the peripheral nervous system, macrophages and Schwann cells appear to be responsible for Apo-E production and the functions of Apo-E are as follows:

1: Cholesterol uptake: Cholesterol and lipids are taken into the liver via endocytosis of lipoprotein particles via the ApoB/E receptor, and taken up by other tissues via the Apo-E receptor.

2: Remnant uptake: Remnants of chylomicrons and VLDL particles are taken up by the liver via the ApoB/E receptor.

3: VLDL formation: Apo-E is incorporated into to coat of the VLDL particle during it's formation. If insufficient cholesterol is available, Apo-E mRNA and protein are degraded.

4: Local redistribution: Apo-E is used to transport cholesterol during cholesterol homeostasis, and to provide cholesterol and lipids during repair.

There is also evidence that Apo-E/VLDL particles can promote branching in the growing neuron, causing axon to split rather than elongate [Bellosta et al 1995]. Free Apo-E appears to have the ability to enhance Ciliary Neurotrophic Factor (CNTF), hence aiding neuronal survival during stress [Gutman et al 1997]. Several immunomodulatory roles have also been proposed, such as the suppression of TNF-α production by activated glial cells [Laskowitz et al 1997].

Of particular importance here is the upregulation of Apo-E during CNS and PNS repair and MS remission, as seen by increased Apo-E in CSF samples of patients [Rifai et al 1987]. Both the sprouting neuron tip and the myelinating Schwann cell express the LDL receptor in large quantities [Poirier 1994]. In crushed rat sciatic nerves, Apo-E mRNA was seen to be expressed at 300 times the normal level, and the protein to be present at high concentrations in nerve homogenate, in fact representing up to 5% of the total soluble protein in the regenerating nerve segment [Ignatius et al 1986]. Levels peak 7-10 days post injury, and slowly return to normal over the next six weeks. This would indicate increased turnover of lipid and cholesterol locally during the repair of the cell membrane and the myelin sheath.

There is a strong relationship between macrophage cholesterol content and Apo-E synthesis. Cholesterol enrichment of macrophages increases Apo-E mRNA and stimulates protein synthesis and secretion [Mazzone et al 1987]. Neither cholesterol esterification nor cholesterol ester accumulation were required for this increase. In the murine macrophage cell line J774, which constitutively expresses human Apo-E, acetylated LDL does not increase Apo-E mRNA transcription or protein production [Mazzone et al 1992]. However, incubation with human HDL_3 stimulated Apo-E secretion through inhibition of Apo-E mRNA degradation. This effect was observed within 15 minutes of incubation and the influence of altered re-uptake of Apo-E (i.e. variation in LDL receptor concentration) by the cell was discounted. The authors suggest that this would indicate that alteration of sterol content does not regulate macrophage Apo-E production at a translational or post-translational level.

1.5.4 APO-E GENOTYPE AND GENERAL LIPID METABOLISM.

In humans, the Apo-E gene is on chromosome 19, and is normally found in one of three allele forms, although other rarer mutations do exist [Myklebost et al 1984]. These allelic types are designated ε2, ε3 and ε4. In primates besides man, only the ε4 allele is found, leading to the assumption that this is the ancestral form of the gene [Mahley and Rall 1999], although ε3 is generally considered to be the "wild type". Interestingly, patients who have received liver transplants acquire the serum Apo-E phenotype of the donor, suggesting that most circulating Apo-E is synthesised by this organ [Linton et al 1991]

The ε2 allele has a cysteine residue instead of arginine at position 158, and the ε4 allele has an arginine instead of a cysteine at position 112 [Rall 1982]. This mutation has a profound effect on the interaction of the α-helical subunits of the protein [Dong and Weisgraber 1996]. The charge difference that these substitutions cause in the protein allow the phenotypes to be separated by iso-electric focusing. The apparent isoelectric points are: ε2 5.57, ε3 5.80 and ε4 6.03 [Zannis 1986]. These three alleles produce a combination of 6 genotypes: ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4 and ε4/ε4. In

Europe, the incidence of the ε4 allele is highest in northern Europe, and decreases further south [Siest et al 1995], which is a similar distribution pattern to MS in native populations.

A secondary form of Apo-E polymorphism, due to post-translational glycosylation, exists. Apo-E is secreted by the liver as an O-glycosylated protein. Carbohydrate, which is variably sialylated, is attached via threonine residue 194 [Rall 1982]. Alternatively sialylated forms of Apo-E beat additional negative charge and focus at positions anodal to the parental isoform in isoelectric focusing.

Binding assays show the ε2 allele to bind to the LDL receptor at 2% of the normal efficiency [Hui et al 1984]. ε2 allele frequency is associated with type III hyperlipoproteinemia, where it accounts for over 90% of cases. These patients have defective catabolism of Apo-E containing lipoproteins and are prone to accumulate lipoprotein remnant particles in their circulation, leading to elevated cholesterol levels and coronary and peripheral vascular disease [Marin et al 1998].

One study in Sweden on Chronic Ambulatory Peritoneal Dialysis (CAPD) patients found the distribution of phenotypes to be normal, but LDL increased in patients with the ε4 allele [Eggertsen 1993]. There was an increase in LDL cholesterol in plasma, but not on total cholesterol levels. Apo-E ε3 exhibits a preference for HDL particles, with Apo-E ε4 preferring VLDL [Dong 1996]. Polymorphism of Apo-E accounts for around 15% of the genetic variability of plasma cholesterol concentrations [Boerwinkle et al 1987].

1.5.5 APO-E GENOTYPE AND NEUROLOGY.

ε4 allele frequency is associated with a number of neurological disorders, such as Alzheimer's disease [Marz et al 1996], Parkinson's disease [Bon 1999], dementia pugelisticus [Jordan et al 1997], and failure to recover from acute spinal or brain damage [Buttini et al 1999]. In the case of Alzheimer's disease, there is evidence

from in vitro studies that Apo-E ε4 can aggregate amyloid protein to form the cerebral plaques indicative of the condition [Sheng et al 1996], but in all cases there is a failure of Apo-E to function correctly in the provision of lipid and cholesterol during nerve repair due to a change in affinity for the Apo-E receptor, and a consequent inhibition of regeneration. Differing levels of Apo-E expression in different parts of the brain may also account for the highly localised nature of Alzhemiers in the brain [Xu et al 1999].

There is also a suggestion of some sort of antioxidant role for Apo-E in neurons, with Apo-E ε4 being the least efficient, and Apo-E ε2 being the most efficient forms of the protein in this regard [Miyata et al 1996]. It may also protect again 4-hydroxynonenal (HNE) induced apoptosis through binding of the HNE at the cysteine residues [Pedersen et al 2000]. Hence a greater neuroprotective effect is seen in the order ε2>ε3>ε4, the same order as the number of cysteines present (ε2=2, ε3=1, ε4=0). Other studies have shown that Apo-E ε4 fails to promote the neurite extension found with Apo-E ε3 [Nathan et al 1994].

Opinion is divided on the detrimental effects of the Apo-E e4 isoform on the various demyelinative disorders. [Chapman et al 1999], [Evangelou et al 1999], [Olivieri et al 1999] and [Gaillard et al 1998] all claim a link between the ε4 allele and progression of disability or cognitive impairment in MS patients. [Weatherby et al 2000], [Ferri et al 1999] and [Rubinsztein 1994] have research suggesting no relationship. Amyotrophic Lateral Sclerosis (ALS) and other demyelinative diseases have also been linked [Moulard et al 1996]. Although no relationship has ever been found between MS incidence and Apo-E genotype, research has been completed on the epidemiological relationship between Apo-E genotype and speed of progression along a disability scale once the disease has been diagnosed.

1.6 CELL TYPES FOR INVESTIGATION.

1.6.1 THE HEPATOCYTE.

Being the primary source of Apo-E in the body, the liver cell is the logical place to start an investigation of Apo-E production. Anti-inflammatory steroids such as the synthetic glucocorticoid dexamethasone significantly increase the mRNA levels of the lipoproteins, including Apo-E [MacMahon 1996], which seem to indicate a role for increase in Apo-E production in the anti-inflammatory environment. Also, for establishment of techniques, it was useful to have a ready source of Apo-E mRNA and protein.

The HepG2 cell line used was a human hepatocyte carcinoma, established from a 15 year old male Caucasian.

1.6.2 THE ASTROCYTE.

The astrocyte is a type of glial cell found in conjunction with neurons throughout the CNS. Like other glial cells, they are unique in producing Glial Fibrillary Acid Protein (GFAP). It consists of a central body and a number of elongated processes that form interfaces with other astrocytes and neurons. The astrocyte interacts with the neuron by performing a limited stimulatory function through the actions of interferons and leukotrienes [Vernadakis 1996]. They also provide a structural framework and regulate ion and metabolite concentrations [Chew and Gallo 1998].

In long term co-cultures of astrocytes and neurons, there is almost complete inhibition of myelin forming activity, even though they do not inhibit axon growth [Ishikawa et al 1996]. In vivo, however, astrocytic scarring, or astrogliosis, similar to that formed by lymphocytes, is a hindrance to regeneration. It must be noted, however, that most CNS cells do not have a great ability to regenerate in the adult in any case [Tetzlaff et al 1994]. Many other substances that are inhibitory to astrocytic activity are also

found in the MS environment, such as the myelin breakdown products NI250 [Bandtlow 1993], and Myelin Associated Glycoprotein (MAG) [Tang et al 1997]. A range of inhibitory proteoglycans are also present [Fawcett and Asher 1999].

Since the astrocyte is also shown to produce Apo-E in situ and in primary cultures [Roses 1996], it is also involved in cholesterol homeostasis and repair if the neuron becomes damaged. The influence of a number of cytokines has also been established on primary human fetal astrocytes [Baskin et al 1997]. Astrocytoma cell lines, such as CCFSTTG1, have been shown to produce Apo-E, and this production can be modulated by insulin, glucagon, or lipid addition [Krul and Tang 1992].

The astrocyte responds to proinflammatory stimuli, such as IFN-γ and TNF-α similarly to neurons by producing cellular adhesion molecules and chemotactic factors, and also by producing pro-inflammatory cytokines of it's own [Aloisi et al 1992]. It responds to anti-inflammatory cytokines by downregulation of these proteins, and IFN-β has been shown to counteract IFN-γ induced astrocyte proliferation [Satoh et al 1996]. It would be reasonable to assume that Apo-E, required by the astrocyte for uptake and redistribution of lipids and cholesterol during degeneration and repair of neurons, would also be modulated by such cytokines.

The locally available human (1321N1) and rat (C6) cell lines were investigated. It was expected that the 1321N1 line would approximate the similar CCF-STTG1 line. Despite the activity of most cytokines to be considered species specific, it was thought useful to have more than one astrocytoma cell line available. The polyclonal Apo-E antibodies currently available commercially exhibit cross reactivity for human and rat Apo-E protein. Also, the gene homology between the species is good enough for rat Apo-E cDNA to function perfectly in detection methods for human Apo-E mRNA.

The 1321N1 cell line used was a human brain astrocytoma, and the C6 cell line was derived from an induced rat glial tumour.

1.6.3 THE MONOCYTE.

The monocyte, or peripheral blood mononuclear cell, is a blood cell that accounts for about 10% of all white blood cells [Health on the Net Foundation]. It commonly circulates in the bloodstream in the form of a clone, a clump of identical cells from a common progenitor. Its function is similar to that of the lymphocyte, in phagocytosis of cells it perceives to be either foreign or infected in some way, but it also functions in recycling cell breakdown products for the regeneration of tissues.

Peripheral blood monocytes secrete prostaglandin [Aberg 1990] before MS relapses and are known to be involved in demyelination in MS, but do not form scar tissue that can prevent regrowth of neurons. Since it produces large amounts of Apo-E, third only to the liver and astrocyte, it is obviously involved in local redistribution of cholesterol and lipid. This production of Apo-E is known to be downregulated, by transcriptional, translational, and post-translational methods, by pro-inflammatory cytokines [Brand et al 1993]. Apo-E and LDL expression by the macrophage can also be regulated by sterol [Mazzone and Basheeruddin 1991]. It could therefore be assumed that Apo-E production is either upregulated by anti-inflammatory cytokines, or at least the Apo-E inhibition could be relieved.

It is worth noting, however, that inflammatory cytokine mRNA production in monocytes is unaffected by IFN-β-1b [Byskosh and Reder 1996], unlike in the T-lymphocyte, so any effects exerted via the monocyte are due to the modulation of other proteins. It must also be considered that the activated monocyte, which begins to differentiate into the macrophage, has an even greater output of Apo-E [Basheeruddin et al 1992].

Monocytes from HIV and Hepatitis-C screened buffy coats, obtained from the Blood Transfusion Service Board, were investigated. Also, several transformed monocyte cell lines were established, with the THP-1 line being the most productive.

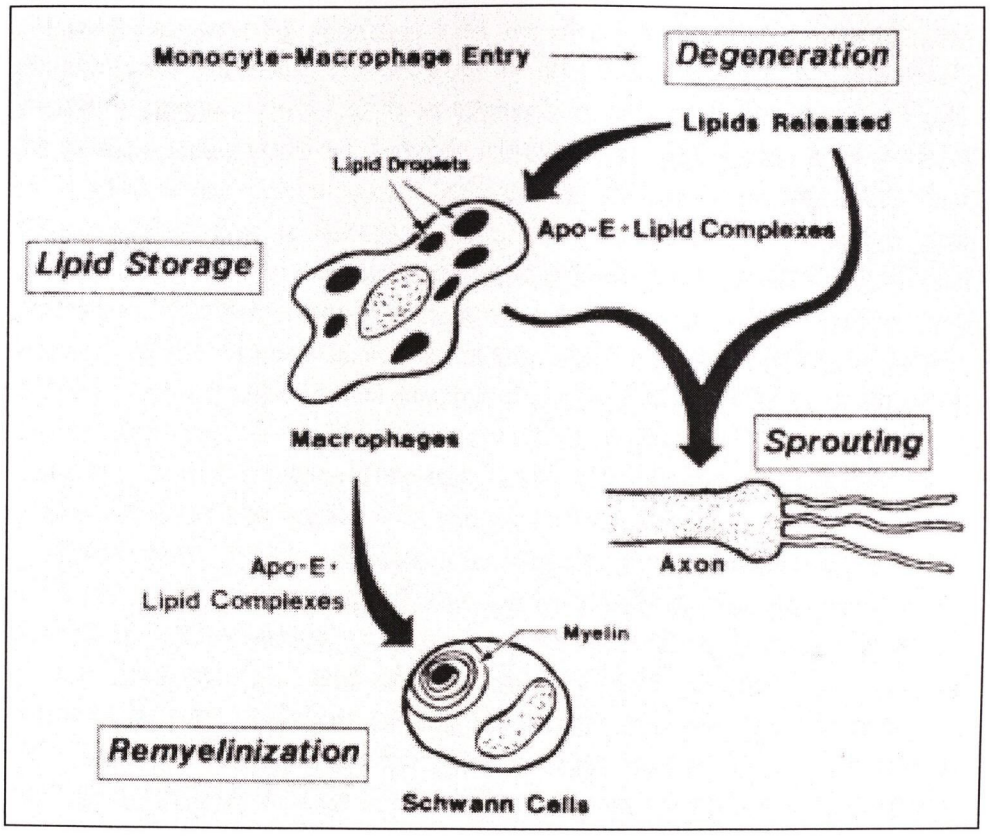

Figure 1.4: Proposed model for macrophage support of neuronal regeneration: *A model for the recycling of lipids and cholesterol after a neurodegenerative attack, in which the macrophage scavenges neuronal breakdown products and secretes Apo-E containing lipid complexes, available to the growing axon.* [Mahley 1988]

The THP-1 cell line was derived from the peripheral blood of a 1 year old male with acute monocytic leukemia. Upon activation with lipopolysaccharide or phorbol myristate acetate (PMA), they differentiate into adherent, macrophage-like cells.

1.7 OUTLINE OF THE STUDY.

The Apo-E genotype of the astrocyte or macrophage may give some indication of the ability of the individual to perform regeneration of damaged neurons arising from a relapse of MS, particularly under the influence of IFN-β medication. Obviously, Apo-E genotyping would be much easier to perform on the blood resident macrophage than the brain resident astrocyte.

Thus the aims of the project are:

1: To set up a model, using an astrocytoma cell line, for measurement of Apo-E mRNA and protein.

2: To attempt to modify the production of Apo-E mRNA and protein using anti-inflammatory cytokines and/or pro-inflammatory cytokines.

3: To isolate macrophages from normal and patient blood and repeat the above, with genotyping of each sample.

4: To assess if the response of macrophage cell Apo-E mRNA and protein to cytokines is a function of the Apo-E genotype.

5: To assess, using EDSS and/or MRI, if efficacy of IFN-β treatment is related to Apo-E synthesis and genotype.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS

MATERIAL	SOURCE
Bacterial culture solutions	Difco
Agarose	Sigma
DIG labelling and detection kit	Boehringer Mannheim
+ve charged nylon membranes	Boehringer Mannheim
Cell lines	E.C.A.C.C.
Cell culture wares	Greiner
DMEM	Sigma
RPMI-1640	Sigma
FCS	Gibco
Histopaque 1077	Sigma
IFN-γ, TNF-α	R&D
TGF-β	Sigma
IFN-β	Schering
PMA	Sigma
^{35}S labelled methionine	Amersham
Rabbit anti human Apo-E antibody	Dako
Protein A Sepharose	Sigma
Acrylamide	National Diagnostics
PPO	BDH
RNA Isolator	Genosys
Ribogreen kit	Molecular Probes
Centricon 10	Amicon
BSA	Sigma
Nitrocellulose	Sigma
3mm blotting paper	Whatman
Goat anti rabbit - HRP conjugate	Sigma
Reverse Transcriptase kit	Promega

SYBR Green kit	Promega
PCR primers	MWG Biotech
Restriction endonucleases (Cfo I, BamH1, Hind III)	Boehringer
LDH cytotoxicity assay kit	Promega

Plasmid pAE155 containing the cDNA clone for Apo-E was a gift from B. Stales, Laboratorium voor Expermentele Geneeskunde en Endocrinologie, Gathuisberg, Katholieke Universiteit Leuven, Belgium. β-actin cDNA was a gift from F. Martin, Department of Pharmacology, University College Dublin. Human Apo-E genotypic standards were a gift from Dr. Philip Wenham, Department of Clinical Chemistry, Western General Hospital, Crew Road, Edinburgh, Scotland.

All other chemicals were analytical grade and were obtained from Sigma or BDH.

Amicon	Amicon Ltd., Upper Mill, Stonehouse, Glos.,GL10 2BJ, U.K.
Amersham	Amersham International plc., c/o P.J. Brennan & Co., Stillorgan, Co. Dublin.
BDH	BDH Chemicals Ltd., c/o Lennox Chemicals, J.F. Kennedy Drive, Dublin 12,

Boehringer	Boehringer Mannheim U.K., (Diagnostics & Biochemicals) Limited, Bell Lanes, Lewes, East Sussex, BN7 1LG, U.K.
DAKO	DAKO Ltd., Denmark House, Angel Drove, Ely, Cambridge, CB7 4ET, U.K.
Difco	c/o Unitech Ltd., Belgard Rd., Dublin 24
E.C.A.C.C.	E.C.A.C.C. PHILS CAMR, Porton Down, Salisbury, Wilts., SP4 OJG, U.K.
Genosys	Genosys Biotechnologies Inc., 162A Cambridge Science Park, Milton Rd., Cambridge, CB4 4GH, U.K.
Gibco	Life Technologies Ltd., 3 Fountain Drive, Paisley, PA4 9RF, U.K.
Greiner	Greiner GmBH, Maybachstrasse 2, D-7443 Frickenhausen, Germany.

Molecular Probes	4849 Pitchford Ave., Eugene, OR., 97402-9165, USA.
MWG-Biotech	MWG-Biotech U.K. Ltd., Waterside House, Peartree Bridge, Milton Keynes, MK6 3BY, U.K.
National Diagnostics	c/o I.S.I.S., UD 11, Southern X Business Park, Bray, Co.Dublin.
Pharmacia	Pharmacia Fine Chemicals Ltd., Mid Summer Boulevard, Milton Keynes, MK9 3HP, U.K.
Premier Brands	Premier Brands U.K. Ltd., P.O. Box 171, Birmingham, B30 2NA, U.K.
Promega	Promega Corp., c/o Medical Supply Co. Ltd., Unit 9, Santry Industrial Estate, Dublin 9.
R&D	R&D Systems Europe Ltd., 4-10 The Quadrant, Barton Ln., Abingdon, Oxon, OX14 3YS,U.K.

Schering	Schering Plough Ltd., Brinny, Inishannon Co. Cork
Sigma	Sigma Chemicals Co. Ltd., Fancy Rd., Poole, Dorset, U.K.
Whatman	Whatman Ltd., Maidstone, U.K.

METHODS

2.2 cDNA PROBE CONSTRUCTION.

Adapted from the method of [Dagert and Ehrlich 1979].

2.2.1 COMPETENT E.COLI PREPARATION.

Competent E.Coli cells were prepared by transferring a single colony of E.Coli HB101 from an agarose plate into 10mL of Luria Broth (LB) in a loosely capped sterilin. The sterilin was incubated for 8 hrs at 37°C in an incubation shaker at 300 cycles per minute. The contents of the sterilin were transferred to 500mL of Luria Broth in a baffled 2L flask, and incubated at 37°C in the incubation shaker at 300 cycles per minute, until the optical density of the culture was 0.2 absorption units at 600 nanometres. The broth was transferred to 250mL centrifuge tubes and centrifuged at 6000 RPM for 10 minutes at 4°C in a Sorvall 5C5C Plus centrifuge. The supernatant was discarded, and the cellular pellet was resuspended in 200mL of ice cold 0.1M $CaCl_2$ for 20 minutes. The suspension was transferred to 50mL centrifuge tubes and centrifuged at 6000 RPM in a Sorvall RC5C centrifuge for 10 minutes at 4°C. The supernatant was discarded, and the cellular pellet was resuspended in 10mL of ice cold 0.1M $CaCl_2$. The suspension was stood on ice for 6 hours. 2mL of sterile glycerol were added, and the solution mixed by gentle inversion. 0.5 mL aliquots were stored at -80°C in cryogenic freeze tubes for later use.

2.2.2 TRANSFORMATION OF COMPETENT CELLS.

Frozen competent cells were thawed for 30 minutes on iced water. 10µL of the relevant plasmid preparation was added to 100µL of cells, and the suspension stored on ice for 1 minute. The suspension was heat shocked on a water bath to 37°C for 1 minute.500µL of LB, 10mM $MgCl_2$, was added and the tube incubated at

Table 2.1: Characteristics of Apo-E and β-actin cDNA containing plasmids.

cDNA	Vector	Length (base pairs)	Site of cDNA probe	Endonuclease used to excise	Antibiotic resistance	Buffer system
Apo-E	pAE155	1100	Pst 1	Linearise with Bam H1	Tet	A
β-actin	pBR322	1200	Hind III	Hind III	Amp	B

Buffer system A contains 50mM Tris acetate, 1mM DTE, 10mM $MgCl_2$. 100mM NaCl, pH 7.5. Buffer system B contains 10mM Tris acetate, 5mM $MgCl_2$. 100mM NaCl, pH 8.0. Both Apo-E and β-actin containing plasmids are derivatives of the plasmid pBR322. The Apo-E cDNA probe is in the Pst 1 site of the plasmid pAE155, but cannot be excised from this site as the insert itself contains Pst 1 sites and would be digested.

37°C for 50 minutes. 200µL aliquots were plated onto Luria Broth agar containing the relevant antibiotic:

Apo-E plasmid: Tetracycline at 10µg/mL

β-actin plasmid: Ampicillin at 60µg/mL

Plates were incubated at 37°C until at least 10 colonies were visible, usually 48 hours. Expected transformation frequency with the plasmid pAE155 can be calculated as follows:

$$\text{Transformations expected} = \frac{\text{no. colonies grown on LB plate}}{\text{mg DNA in transformation mix}}$$

$$= 2 \times 10^7 \text{ per mg pAE155}$$

2.2.3 STORAGE OF E. COLI CONTAINING APO-E cDNA CLONES.

An LB agar plate was streaked with stock culture and grown overnight at 37°C. Approximately 50 colonies were scraped from the plate and used to inoculate 2mL of 2% Bacto peptone solution in a 5mL Bijou tube. 2mL of glycerol (99% w/v) was added, vortexed, and the culture stored at -20°C.

2.2.4 ISOLATION OF PLASMID DNA.

Using a sterile platinum loop, a single colony from each plate was used to inoculate 200mL of LB containing the relevant antibiotic, as follows:

Apo-E plasmid: Tetracycline at 10µg/mL

β-actin plasmid: Ampicillin at 60µg/mL

Flasks were incubated at 37°C in the incubation shaker at 37°C at 600 cycles per minute, until the optical density of the culture was 0.2 at 600 nm. The culture was transferred to 250mL centrifuge tubes and centrifuged for 15 minutes at 4°C at 5000g in the Sorvall RC5C Plus centrifuge. The supernatant was discarded, and the cellular pellet was resuspended in 6mL of freshly prepared 25mM Tris-Cl, pH 8.0, 10mM EDTA, 15% Sucrose, 2mg/mL lysozyme. The suspension was incubated on iced water for 10 minutes, and 12mL of 0.2M NaOH, 1% SDS, were added. The tube was mixed by inversion and incubated on iced water for 10 minutes. 7.5mL of 3M Sodium Acetate, pH 4.6, was added, and mixed by inversion. The tube was incubated on iced water for 20 minutes. The lysed cells were transferred to 50mL centrifuge tubes and centrifuged at 33,000g at 4°C for 15 minutes. The supernatant was transferred to a clean tube, and 50µL of 1mg/mL RNAase added and mixed by inversion. An equal volume of phenol:chloroform:isoamyl alcohol, 20:19:1, was added and mixed by inversion, and the tube incubated on ice for 30 minutes. The tube was centrifuged at 10,000 RPM at 4°C for 20 minutes, and the upper layer transferred to a clean tube. The phenol/chloroform extraction was repeated, and an equal volume of chloroform added to the retained upper layer, and the tube incubated on ice for 20 minutes. The tube was centrifuged at 10,000 RPM at 4°C for 20 minutes, and the upper layer transferred to a clean tube. The solution was washed with 2 volumes of 100% ethanol and incubated on ice for 20 minutes, and centrifuged at 10,000 RPM at 4°C for 20 minutes. The supernatant was discarded, and the DNA pellet resuspended in 1.6mL dH_2O, 0.4mL 4M NaCl, 2mL 13% polyethyleneglycol (8000 MW). The tube was incubated on ice for 60 minutes, and centrifuged at 12,000g at 4°C for 15 minutes. The pellet was washed once with 5mL of 70% ethanol, and centrifuged at 15,000g at 4°C for 5 minutes. The plasmid pellet was dried under air and resuspended in 100µL of 10mM Tris-Cl, 0.1mM EDTA, pH8.0 (TE). 10µL of the plasmid preparations were diluted with 990µL of TE and absorbancy measured in a spectrophotometer at 260 and 280 nanometers. A 260/280 reading of 1.8 to 2.0 indicated high quality DNA, and the concentration was assessed using the formula:

ABS 260nm x dilution factor x 50 = µg/mL DNA.

2.2.5 PREPARATION OF cDNA.

10μL of plasmid preparation was added to 10μL of 1X restriction endonuclease buffer and 2μl of the relevant restriction endonuclease was added:

Apo-E plasmid: Bam H1
β-actin plasmid: Hind III

The tubes were incubated at 37°C overnight. 10μL of DNA loading buffer was added to each sample and incubated at room temperature for 30 minutes. 20μL of each sample was then added to each well of a 1% agarose, Tris-Cl/Acetic acid/EDTA gel, and run in 1X TAE buffer at 100V for 2 hours. The gel was stained for 1 hour in 1mg/mL ethidium bromide solution, and destained overnight in distilled water. The DNA was visualised under UV light and the relevant bands cut from the gel. Gel segments were centrifuged for 10 minutes in GenElute spin columns to remove agarose. 10μL of the cDNA preparations were diluted with 990μL of TE and purity and concentration measured using absorbancy in a spectrophotometer at 260 and 280 nanometers.

2.2.6 DIG LABELLING OF cDNA.

1μg of cDNA was made up to 16μL with dH_2O, and heat denatured in a boiling water bath at 100°C for 10 minutes. The sample was transferred on to iced water, and 4μL of DIG High Prime (Boehringer Mannheim) labelling mixture was added and incubated overnight at 37°C. The reaction was stopped using 2μL of 200mM EDTA, and the probes diluted to 5mL with High SDS hybridisation buffer to a concentration of 0.5ng/mL for Apo-E and 10ng/mL for β-actin. Probes were stored at -20°C for later use.

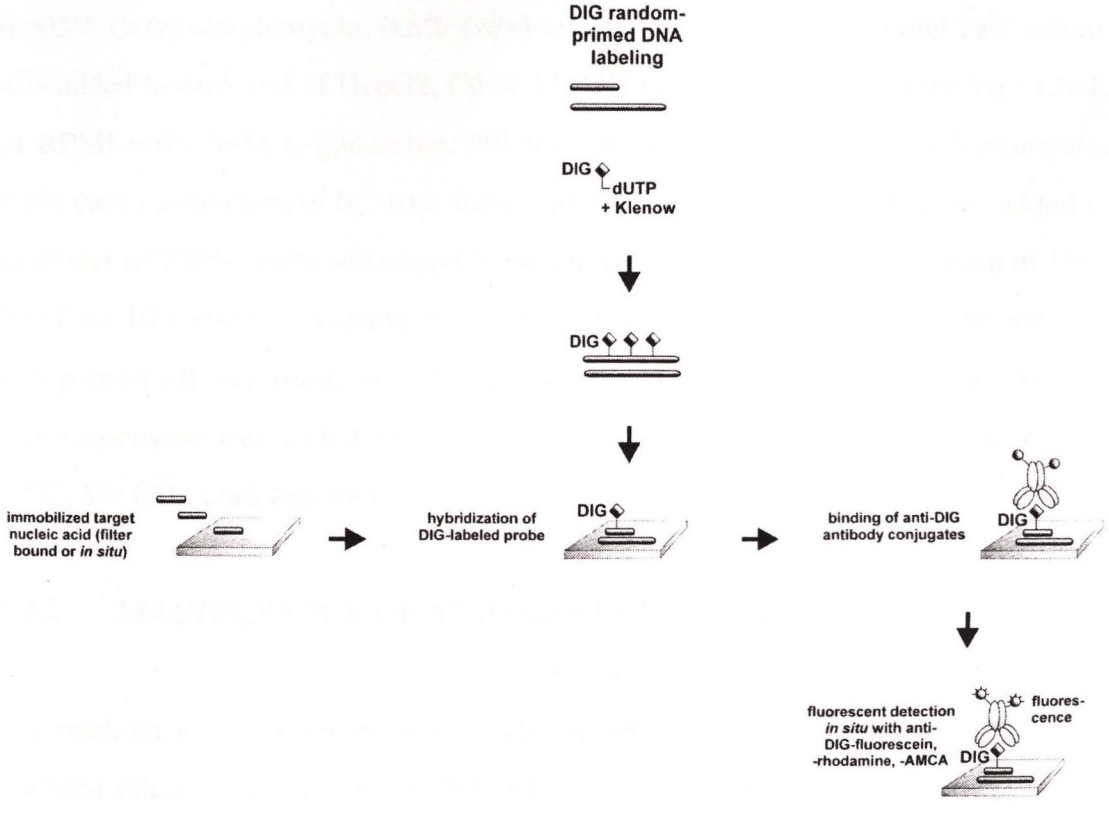

Figure 2.1: Dig labelling and detection protocol: *DIG labelled fragments of cDNA are generated, and are then allowed to hybridise with RNA that has been bound to a nylon membrane. After washing steps, an anti-DIG antibody attached to an enzyme is allowed to bind. This enzyme can use a substrate, such as CSPD, to produce light.*

2.3 CELL LINE CULTURE.

2.3.1 REVIVAL OF FROZEN CELL LINES.

A vial of each cell type was removed from liquid nitrogen storage and the contents added to a sterilin. 12mL of DMEM with 2mM L-glutamine, 50U/mL penicillin, 0.005% (w/v) streptomycin, 0.8% (w/v) amphotericin B, and 5% foetal calf serum, was added to each vial of HepG2, C6 or 1311N1 cells and mixed by pipetting. 12mL of RPMI with 2mM L-glutamine, 50U/mL penicillin, 0.005% (w/v) streptomycin, 0.8% (w/v) amphotericin B, 10% foetal calf serum and 20mM HEPES was added to each vial of THP-1 cells and mixed by pipetting. The tubes were centrifuged at 1000 RPM for 10 minutes to remove the DMSO or glycerol cryoprotectant. The medium was poured off and 10mL of fresh medium added and mixed by pipetting. 5mL of this suspension was added to each of two $25cm^2$ flasks, which were incubated at 37^oC, 5% CO_2, until required.

2.3.2 MAINTENANCE OF ADHERENT CELL LINES.

The medium was removed from the cells, and the cells washed once with phosphate buffered saline (138mM NaCl, 2.6mM KCl, 1.5mM KH_2PO_4, 8.2mM Na_2HPO_4. 3mL of 0.5% (w/v) trypsin, 0.2% EDTA (w/v) solution was added to each flask and incubated at 37^oC for 10 minutes. 9mL of medium was added to the flask, and the contents transferred to a fresh sterilin, which was centrifuged at 1000 RPM for 5 minutes. The medium was discarded, and 100µL of medium was added to each well for experimentation. 100µL of resuspended cells was added to 1.9mL of medium in each well of a 6 well plate, and returned to the incubator until confluent.

2.3.3 MAINTENANCE OF SUSPENSION CELL LINES.

The entire contents of one $75cm^2$ flask (c. 30mL) were mixed by pipetting and transferred to a sterile 50mL tube. The tube was centrifuged at 1000 RPM for 5

minutes, and the medium discarded. 30mL of RPMI with 2mM L-glutamine, 50U/mL penicillin, 0.005% (w/v) streptomycin, 0.8% (w/v) amphotericin B, 10% foetal calf serum and 20mM HEPES was added to the tube, and mixed by pipetting. 5mL of this suspension were added to 25mL of fresh medium in a 75cm^2 flask and returned to the incubator.

2.3.4 LONG TERM STORAGE OF CELLS.

Subconfluent cultures of cells (<2x10^4/cm^2) cells per were trypsinised if necessary, and counted using Trypan Blue stain and a haemocytometer. For adherent cell types, equal volumes of cell suspension containing approximately 2 x 10^6 cells, and dimethyl sulfoxide (DMSO) (10% v/v)containing 40% FCS in DMEM were mixed together. For THP-1 cells, for which DMSO is extremely toxic, equal volumes of cell suspension containing approximately 2 x 10^6 cells, and glycerol (10% v/v) containing 40% FCS in DMEM were mixed together. The cryoprotectant was added dropwise, with mixing of the universal after each addition. No more than half the volume of cryoprotectant was added in the first minute. When all was added, the universal was mixed well, and the cell suspension was dispensed into cryo-freeze vials. Each vial was frozen to -20^{o}C for 1hr, then to -70^{o}C for 1hr, before being immersed in liquid nitrogen. One vial was thawed the following day to check that the cells had retained viability.

2.4 PRIMARY CELL CULTURE.

2.4.1 ACQUISITION OF BUFFY COATS.

Identical blood group buffy coats were received from the Blood Transfusion Service. These samples were from random anonymous donors, and represented the leukocyte rich fraction remaining after centrifugation of 500mL of blood to separate plasma from blood cells. Buffy coats were commonly collected after storage overnight at

37°C in sterile, heparin anti-coagulant coated bags. All samples received had passed primary screening for HIV and Hepatitis B, and the volume was generally 50mL.

2.4.2 ISOLATION OF LEUKOCYTES.

In a 50mL tube, 20mL of buffy coat was carefully overlaid onto 20mL of Histopaque-1077 (Sigma). Tubes were centrifuged at 2250 RPM for 30 minutes in a benchtop centrifuge, as in Figure 2.2, and the white cell layer removed to a fresh tube. 200µL of the plasma layer was retained for use as a standard in Western blotting. 20mL of 10% FCS, RPMI-1640 was added to each tube and aspirated, and the tubes centrifuged at 1550 RPM for 10 minutes. This washing step was repeated, and the cellular pellet resuspended thoroughly in 10mL of 10% FCS, RPMI-1640.

2.4.3 ISOLATION OF PBMCs.

An aliquot of the leukocyte suspension was diluted 1:10 with PBS, and incubated with an equal volume of 0.4% Trypan Blue for 10 minutes. 10µL of this suspension was used in conjunction with a haemocytometer to count cell numbers. Cell density was adjusted to $1x10^7$ cells per mL with 10% FCS, RPMI-1640, and 1mL added to wells of activated 24 well tissue culture plates. These plates have been treated with a cell attachment ligand of human fibronectin so there is no waiting for the attachment matrix to deposit. Cells were incubated at 37°C, 5%CO_2, for 1 hour. The non-adherent cell suspension was removed, and the cells washed vigorously three times with PBS, fed with 2mL of 10% FCS, RPMI-1640, and returned to the incubator.

2.5 TREATMENT OF CELLS.

2.5.1 TREATMENT OF ADHERENT CELLS.

The cells were pre-treated with 1mL of DMEM with 2mM L-glutamine, 50U/mL penicillin, 0.005% (w/v) streptomycin, 0.8% (w/v) amphotericin B, 20mM HEPES

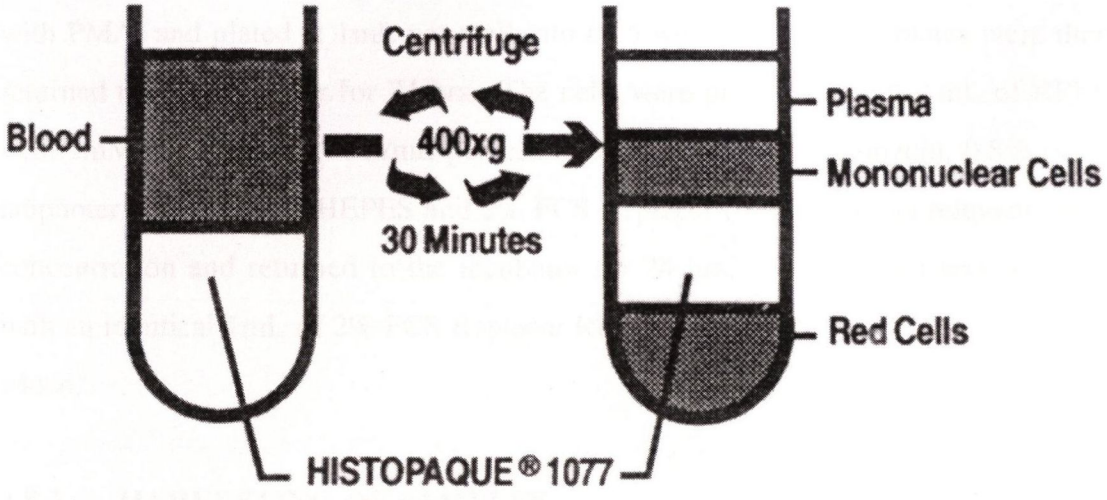

Figure 2.2: The separation of leukocytes from red blood cells by Histopaque 1077:

After centrifugation these layers are observed. If the plasma or Histopaque layers contain red blood cells then the gradient has been overloaded and must be repeated.

and 2% FCS Replacer (Sigma), and returned to the incubator for 24 hrs. The medium was replaced with an identical 1mL of 2% FCS Replacer DMEM and the cytokines for investigation added.

2.5.2 TREATMENT OF MONOCYTE CELLS.

For THP-1 cells, 25mL of cell suspension was activated by making to 10^{-7}M with phorbol 12-myristate 13-actetate (PMA), and plated at 1mL per well into a 25 well plate. For primary cells, 25mL of cell suspension was activated by making to 10^{-8}M with PMA, and plated at 1mL per well into a 25 well plate. These plates were then returned to the incubator for 24 hrs. The cells were pre-treated with 1mL of RPMI with 2mM L-glutamine, 50U/mL penicillin, 0.005% (w/v) streptomycin, 0.8% (w/v) amphotericin B, 20mM HEPES and 2% FCS Replacer (Sigma), at the relevant PMA concentration and returned to the incubator for 24 hrs. The medium was replaced with an identical 1mL of 2% FCS Replacer RPMI and the cytokines for investigation added.

2.5.3 HARVESTING OF SAMPLES.

Medium was removed and frozen to -80^{o}C if not used immediately. Cells were then washed once with PBS, and 1mL of RNA isolation fluid (Genosys) was added to each well. Samples were homogenised by pipetting, and frozen at -80^{o}C or used immediately.

2.6 ^{35}S LABELLING OF CELLS.

^{35}S is a weak β radiation emitter, and has a half-life of 87.1 days. The isotope is incorporated into proteins as ^{35}S-labelled methionine, which is an essential amino acid for mammalian cells and must therefore be supplied in the medium.

2.6.1 TREATMENT OF CELLS WITH ^{35}S METHIONINE.

A 24 well plate of 1321N1 cells was allowed to grow to confluency, the medium removed, and washed once with sterile PBS. 1mL of methionine free DMEM with 2mM L-glutamine, 50U/mL penicillin, 0.005% (w/v) streptomycin, 0.8% (w/v) amphotericin B, and 5% foetal calf serum was added, to each well to starve the cells of methionine, for 24 hrs. The medium was removed, 250µl of identical medium containing 200µCi/mL of ^{35}S methionine and 1µM cold methionine fed to each well, and the cells returned to the incubator.

2.6.2 EXTRACTION OF CELLULAR AND MEDIUM PROTEIN.

When ready to be harvested, the medium was removed from the cells and kept in a fresh tube. 250µL of medium with 1µM cold methionine was added to each well and returned to the incubator for 6 hrs to allow all incorporation into proteins to continue until completion. This 250µL was added to the first sample taken. The cells were washed twice with sterile PBS, and homogenised with 100µL of 1% SDS, through pipetting and freeze/thaw cycling. All samples were then stored at -20°C for later analysis.

2.6.3 SCINTILLATION COUNTING.

Medium and cellular protein samples were thawed, vortexed, and 5µL of each added to 10mL of Ecoscint scintillation fluid in a plastic disposable scintillation vial. These samples were read in a Packard TriCarb 2100TR Liquid Scintillation Analyser as Counts Per Minute (CPMs) over the course of three one minute intervals. The mean of these results was calculated and recorded.

2.6.4 IMMUNOPRECIPITATION OF APO-E.

In order to assess what volume of antiserum had to be added to the samples to ensure complete precipitation of Apo-E containing particles, different amounts of antiserum (2-50μL) were added to 100μL of 1321N1 medium. Following the removal of the protein A-Sepharose, an aliquot of each was removed and subjected to SDS-PAGE followed by Western blotting. The volume of antiserum that was shown to completely remove Apo-E from the medium, 10μL, was used in all further experiments.

Medium and cellular protein samples were thawed, vortexed, and 10μl of rabbit anti-human Apo-E antiserum (Dako) added to each sample. Tubes were left on a roller at room temperature for 1 hr. The protein A-Sepharose conjugate was vortexed briefly, and 10μL added to each tube. Tubes were then returned to the roller for 1 hr. For precipitation, all tubes were centrifuged in a benchtop minifuge for 1 minute. Supernatant was removed and stored at -20^{o}C for later analysis. 1mL of PBS, 0.3% Tween-20, was added to each tube of precipitant, vortexed briefly, and left on the roller for 10 mins. The tubes were centrifuged again for 1 min, and the PBS-Tween removed. This washing step was repeated 5 times.

2.6.5 SDS-PAGE ELECTROPHORESIS OF PROTEIN.

15% Polyacrylamide, SDS gels were prepared from stock solutions of 30% acrylamide and 1% N,N'-methylene-bis-acrylamide, with 5% stacking gels portions. Aliquots of total raw medium (10μL), raw cellular extract (10μL), and total immunoprecipitant from medium and cellular protein were mixed with 10μL of loading buffer (0.125M Trizma-HCl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue), and heated to 100^{o}C for 2 minutes on a heating block. Gels were loaded into an Atto Mini-Gel apparatus, and run in PAGE running buffer (0.2M glycine, 0.2M Trizma-HCl, 1% SDS), for 2.5 hours at 100V.

2.6.6 STAINING OF GELS FOR TOTAL PROTEIN.

Gels were removed from plates, and rinsed with distilled water. Stacking gels were cut away, and the resolving gel stained in 1.25% (w/v) Coomassie Brilliant Blue R in 50% (v/v) methanol, 10% (v/v) acetic acid for 10 minutes. The gel was then destained overnight in 10% (v/v) methanol, 10% (v/v) acetic acid. The stained proteins were photographed and noted.

2.6.7 PPO VISUALISATION OF LABELLED PROTEINS.

Gels were rinsed in distilled water and placed into 100mL of DMSO for dehydration for 1 hr in a fume cupboard. This step was then repeated twice with fresh DMSO. A solution was prepared of 22% (w/v) 2, 5-diphenyloxazole (PPO) in DMSO, and the gel transferred into this and left overnight to allow the scintillant to be absorbed by the gel. The gel was placed into 100mL of distilled water, which caused the PPO to come out of solution, and placed onto three stacked sheets of Whatman 3mm blotting paper. The gel and paper were placed onto a gel-drying apparatus set at 80^{o}C for 2 hours. Once dried, the gel was fixed firmly into an autoradiograph cassette, and exposed to X-OMAT LS film (Kodak) for 7 days.

2.7 EXTRACTION OF TOTAL RNA.

2.7.1 ELIMINATION OF RNAase ACTIVITY IN EXPERIMENTS.

All tubes and pipette tips were soaked overnight in 0.1% dimethylpyrocarbonate and then autoclaved to destroy RNAases. DMPC was hydrolysed to water and ethanol, which evaporates, by autoclaving. Water was also treated with DMPC for use in dilutions etc. Where apparatus could not be autoclaved, it was rinsed thoroughly with chloroform and allowed to dry in the fume cupboard.

2.7.2 EXTRACTION OF RNA USING RNA ISOLATOR FLUID.

Samples homogenised in RNA Isolator fluid were thawed to room temperature, 0.2mL of chloroform added, mixed by inversion and incubated at room temperature for 20 minutes. Tubes were then centrifuged in a benchtop minifuge (Beckman Minifuge E) for 15 minutes at 4°C. The upper layer was transferred to a fresh tube, and 0.8mL of isopropanol was added and mixed by inversion (lower layer was stored at 4°C for isolation of cellular protein). Tubes were incubated at room temperature for 15 minutes and centrifuged in the minifuge for 10 minutes at 4°C. The supernatant was discarded, and the RNA pellet washed once with 1mL of 75% ethanol and centrifuged for 5 minutes in the minifuge at 4°C. Samples were air dried, and dissolved in 50µL of DMPC treated H_2O by pipetting and incubation to 50°C for 30 minutes.

2.7.3 QUANTIFICATION OF RNA BY SPECTROPHOTOMETRY.

Purity and concentration were assessed using spectrophotometry, at wavelengths 260nm and 280nm, with a 1:100 diluted sample of the RNA solution. 260/280 ratios of 1.8 to 2.0 indicated good quality RNA and concentrations were calculated as follows:

ABS 260 x dilution x 40 = µg/mL RNA

2.7.4 QUANTIFICATION OF RNA BY FLUORESCENCE.

Where RNA yield was expected to be very low, e.g. in primary monocyte experiments, samples were diluted 1:100 with TE assay buffer (10mM Tris-HCl, 1mM EDTA. pH 7.5). 2µl of this sample was mixed with 98µl of Low Range Assay Mix (RiboGreen DMSO stock:TE buffer, 1:2000). RNA standards (0ng/mL, 1ng/mL, 5ng/mL, 25ng/mL and 50ng/mL) were prepared similarly. Samples were then placed in a black walled 96 well plate and transferred to a fluorescent plate reader. The

excitation wavelength was set to 500nm and the emission wavelength to 525nm, and the plates read.

2.7.5 FORMALDEHYDE GEL ELECTROPHORESIS.

2.25g of agarose were added to 109.5mL of DMPC treated H_2O and heated in a microwave oven for 2 minutes. 15mL of MOPS and 24.3mL of formaldehyde were added, and the mixture poured into the gel tray and allowed to cool. 2µg of RNA was added to 20µL of loading buffer (Bromophenol blue 0.25% (w/v), Ficoll-400 15% (w/v)) containing 1ng/mL of ethidium bromide were mixed and incubated at 60°C for 30 minutes. The samples were added to the gel and run in 1X MOPS at 100V for 1hr. The RNA was then visualised under UV light.

2.8 RNA SLOT BLOTTING.

2.8.1 RNA PREPARATION.

RNA was thawed and vortexed briefly. It was then mixed with dilution buffer (DMPC treated H_2O, SSC x 20, Formaldehyde, 10:6:4) to give 200ng, 100ng and 50ng of RNA in 200µL, and incubated at 37°C for 10 mins.

2.8.2 APPLICATION TO SLOT BLOTTER.

The slot blotting manifold was soaked for 30 minutes in 0.1M NaOH and rinsed with DMPC treated H_2O. A sheet of positively charged nylon membrane (Boerhinger Mannheim) and 3 sheets of 3MM blotting paper (Whatman) were soaked in 20X SSC and placed in the manifold. The edges of the manifold and any unused slots were sealed with parafilm. The vacuum pump was attached and started, and the slots washed through twice with 20X SSC. The diluted RNA was applied to the slots, washed through with 20X SSC, and covered with a sheet of dry blotting paper, to prevent dust contamination, for 60 minutes. The membrane was removed and dried at

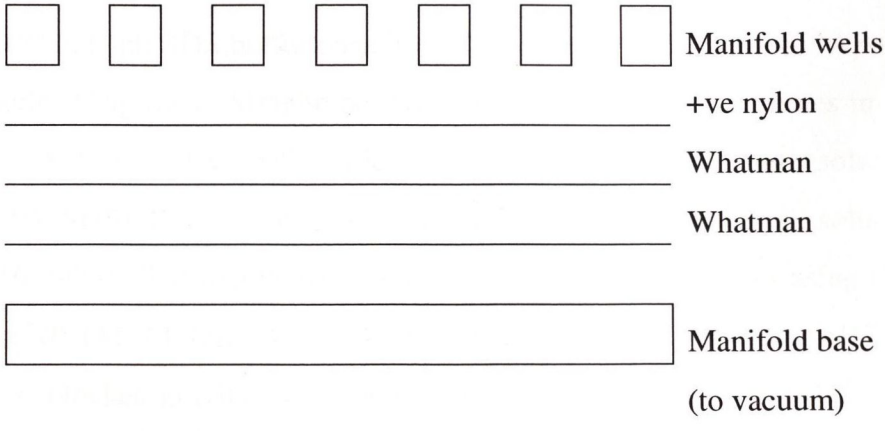

Figure 2.3: Slot blotting apparatus set-up: *A sheet of positively charged nylon (Boehringer), particularly useful for immobilising negatively charged nucleotides, is placed on top of two sheets of 3MM blotting paper (Whatman). These are then placed on top of the slot blotter base, which is attached to a vacuum pump. The manifold lid is screwed tightly into place on top, and the RNA applied into wells.*

100°C for 60 minutes in an oven, and fixed on a UV transilluminator for 2.25 minutes per side.

2.8.3 PROBING WITH DIG LABELLED PROBES.

The membrane was prehybridised in a sterilin at 50°C for 2 hours in a rotating hybridisation oven with High-SDS buffer (7% SDS, 50% Deionised Formamide, 5X SSC, 50mM Sodium Phosphate pH7.0, 0.1% N-Laurylsarcosine, 2% Blocking Reagent (Boerhinger), heated to 68°C). 2 identical membranes were hybridised overnight at 50°C in High-SDS buffer containing DIG labelled cDNA probes (Apo-E: 0.5ng/mL, β-actin: 10ng/mL). Membranes were washed twice for 20 minutes in 2X wash solution (2X SSC, 0.1% SDS), twice for 20 minutes in 0.5X wash solution (0.5X SSC, 0.1% SDS) at 68°C, then twice for 20 minutes in 0.1X wash solution (0.1X SSC, 0.1% SDS). The membranes were then washed for 10 minutes using DIG wash solution (0.1M Maleic Acid, 0.15M NaCl, 0.3% Tween-20, pH7.5). Membranes were blocked in DIG block solution (0.1M Maleic acid, 0.15M NaCl, pH 7.5, 1% Blocking reagent (Boerhinger) for 1 hour, and Anti-DIG peroxidase conjugate solution (0.25µL Digoxigenin Fab (Boehringer) in 5mL of DIG blocking solution) was bound to the membrane for 30 minutes. Blots were washed in DIG wash solution for 20 minutes 3 times, and soaked in DIG detection buffer (100mM Tris-Cl, 100mM NaCl, pH 9.5) for 5 minutes.

2.8.4 VISUALISATION.

CSPD chemiluminescent substrate was applied to each membrane sufficient to soak it completely, and the blot placed between 2 sheets of acetate in an autoradiography cassette at 37°C for 30 minutes. Blots were exposed to photographic film for 30 minutes, developed in an automatic film developer (Fuji) and slot intensities measured using scanning densitometry.

2.9 EXTRACTION OF PROTEIN SAMPLES.

2.9.1 CELLULAR PROTEIN.

The lower layer remaining from RNA isolation was removed from refrigeration, and the DNA and lipid containing interphase entirely removed. 0.8mL of isopropanol was added to each tube and mixed by inversion. Protein was precipitated by centrifugation for 10 minutes in a minifuge at 4°C. The supernatant was discarded, and the protein pellet washed three times with 0.3M guanidinium hydrochloride, 95% ethanol, followed by one wash in 100% ethanol. The protein pellet was resuspended in 50µL of 1% SDS by heating to 100°C for 20 minutes. Insoluble material was precipitated by centrifugation in a minifuge for 10 mins. Supernatants were transferred to fresh tubes and stored at -20°C.

2.9.2 MEDIUM PROTEIN.

Medium was thawed and centrifuged for 5 minutes in a Minifuge to remove cellular debris. The supernatant was centrifuged in a 10000 Dalton MW cut-off Centricon concentrator (Amicon) in a Sorvall RC5C Plus centrifuge at 6360 RPM for 90 minutes. 2mL of dH_2O was added to each tube, and again centrifuged at 6360 RPM for 90 minutes. The concentrated, desalted medium (about 150µL) was transferred to a fresh tube and lyophilised in a Speed-e-Vac for 2 hours. 50µL of 1% SDS was added, and mixed by vortexing. The samples were stored at -20°C.

2.10 PROTEIN QUANTITATION.

0ng, 50ng, 100ng, 200ng and 400ng of BSA, 10µL of cellular protein solution and 1µL of concentrated medium were each made up to 250µL with water. 100 parts Markwell reagent A (2% Na_2CO_3 (w/v), 0.4% NaOH (w/v), 0.16% sodium tartrate (w/v), 1% SDS (w/v)) and one part Markwell reagent B (4% $CuSO_45H_2O$ (w/v)) were mixed, and 750µL added to each sample. Tubes were incubated at room temperature

for 15 minutes, and 75µL of diluted phenol reagent (Folin-Ciocalteau reagent:H_2O, 1:1) added and mixed by vortexing. Tubes were covered and incubated at room temperature for 45 minutes. Absorbance was read in a spectrophotometer at wavelength 660nm.

2.11 SDS-PAGE ELECTROPHORESIS.

2.11.1 SAMPLE PREPARATION.

Aliquots of resuspended cellular protein (20µg) and concentrated medium (50µg) were mixed with 10µL of loading buffer (0.125M Trizma-HCl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue), and heated to 100°C for 2 minutes on a heating block.

2.11.2 ELECTROPHORESIS.

15% Polyacrylamide, SDS gels were prepared from stock solutions of 30% acrylamide and 1% N,N'-methylene-bis-acrylamide, with 5% stacking gels portions. Gels were loaded, and run in PAGE running buffer (see page 55), for 2.5 hours at 100V.

2.11.3 IMMUNOBLOTTING.

Gels were removed from plates, and protein was transferred to nitrocellulose membranes in a Semiphor semi-dry blotter using transfer buffer (0.192M glycine, 25mM Trizma-HCl, pH 8.3, 15% (w/v) methanol) for 90 minutes at 115mA. Membranes were stained for total transferred protein in Ponceau stain and rinsed in dH_2O. Gels were stained overnight in Coomassie blue for untransferred protein and destained with 25% methanol, 5% glacial acetic acid. Blots were then blocked overnight in 3% Marvel TTBS (0.5M NaCl, 20mM Tris-Cl, pH 7.5, 0.05% (v/v) Tween-20), overnight at 4°C. Rabbit anti human Apo-E antibody (Dako) was bound

to the blot for 1 hour at 1:500 concentration in 3% Marvel TTBS, and the blot washed in 0.05% (v/v) Tween-20 PBS for 10 minutes 3 times. Goat anti rabbit peroxidase conjugate (Sigma)) was bound to the blot for 1 hour at 1:2000 concentration in 3% Marvel TTBS, and the blot washed in 0.05% (v/v) Tween-20 PBS for 10 minutes 6 times.

2.11.4 VISUALISATION.

For more sensitive blots, membranes were then soaked in freshly prepared SuperSignal chemiluminescent substrate (Solution A:Solution B, 1:1) (Pierce) for 10 minutes and placed between two sheets of acetate in an autoradiography cassette. Blots were exposed to photographic film for 2 to 10 minutes, and the film developed in a automatic film developer (Fuji).

For less sensitive blots, membranes were washed twice in 0.05% Tween-20 PBS for 10 minutes and soaked in 0.5M NaCl, 40mM Tris-Cl, 0.075% (v/v) hydrogen peroxide, 0.075% (w/v) 4-chloronaphthol, 20% (v/v) methanol, for 30 minutes. Blots were then left overnight in PBS-Tween at $4^{o}C$.

2.12 RT-PCR QUANTITATION OF APO-E mRNA.

2.12.1 GENERATION OF cDNA.

All RNA samples were adjusted to 100pg/µL with DMPC treated water, and 2.5µL added to the following reverse transcriptase mix:

$MgCl_2$:	(25mM)	2µL
Reverse transcription buffer:	(10X)	1µL
dNTPs:	(10mM each)	1µL
Ribonuclease inhibitor:	(40U/µL)	0.25µL
AMV reverse transcriptase:	(20U/µL)	0.375µL

Random hexamer primers:	(500µg/mL)	0.5µL
DMPC water:		2.375µL
Total reaction volume:		10µL

Tubes were incubated at 42°C for 1hr, and reverse transcription halted by heating to 99°C for 5minutes. Tubes were then cooled to 1°C for 5 minutes to inactivate the reverse transcriptase, and the samples used immediately or else stored at -20°.

2.12.2 TAQMAN FLUORESCENT PCR THEORY.

The TaqMan Fluorescent PCR system is a procedure for the quantitative, real-time analysis of the generation of PCR products. Its basis is the SYBR-Green fluorescent dye, which fluoresces only when bound to double stranded DNA, such as that found at the end of the extension step of a PCR cycle. The apparatus is basically a thermal cycler with a fluorescent plate reader built in to the lid, and special optical caps are used for the reaction tubes. The cycle number at which fluorescence crosses a certain threshold gives an indication of the amount of starting template, and therefore an estimation of the amount of RNA used to make that template. SYBR-Green absorbs and emits maximally at 485nm and 520nm respectively.

The DNA polymerase use is AmpliTaq Gold. This is a high grade Taq polymerase enzyme with a bound protein which inactivates it. Heating to 50°C for 2 minutes causes the dissociation of this extra protein and allows polymerisation to proceed. This modification means there is no need to open the PCR tubes to add the Taq, as in a normal PCR using a "hot-start" protocol.

Also included is AmpErase UNG (uracil-N-glycosylase), which is a 26kDa recombinant enzyme which acts on both single and double-stranded DNA by hydrolysing uracil-glycosidic bonds at dUTP-containing DNA sites. This releases the uracil nucleotide, preventing reamplification of carryover PCR products.

2 primers sets were used, one for amplification of Apo-E cDNA, and one for glucose-6-phosphate dehydrogenase (G6PDH) cDNA. G6PDH is a housekeeping gene whose expression is known to be unaffected by most cytokines.

Apo-E forward primer: (E23): 5'(TCCAAGGAGCTGCAGGCGGCGCA)3'
Apo-E reverse primer: (E31): 5'(ACAGAATTCGCCCCGGCCTGGTACACT)3'
G6PDH forward primer: (G6P1): 5'(TTCTTCAACCCCGAGGAGT)3'
G6PDH reverse primer: (G6P2): 5'(GGGAAGGAGGGTGGCCGTG)3'

2.12.3 TAQMAN FLUORESCENT PCR PROTOCOL.

2.5µL of the cDNA generated from reverse transcription was added to the following PCR mix in MicroAmp Optical 96-well reaction plates (Perkin Elmer):

SYBR-Green dye:	(10x)	2.5µl
$MgCl_2$:	(25mM)	3.0µl
dNTPs:	(10µM each)	2.0µl
AmpliTaq Gold:	(5U/µL)	0.125µl
AmpErase UNG	(1U/µL)	0.25µl
Primer 1:	(25µM)	1.0µl
Primer 2:	(25µM)	1.0µl
dH_2O:		12.625µl

Plates were centrifuged at 1000 RPM for 1 min, and bubbles removed with a sterile needle. Optical caps (Perkin Elmer) were firmly affixed, and the plate placed in the GeneAmp 5700 Sequence Detection System (Perkin Elmer). The heating lid/fluorescent reader was screwed into place, and the machine programmed to run the following protocol:

50°C: 2 minutes ->AmpliTaq Gold activation.
95°C: 10 minutes ->PCR "hot-start".

95°C: 30 seconds ->Denaturation.]

65°C: 30 seconds ->Primer annealing.] 40 cycles.

70°C: 90 seconds ->Extension.]

70°C: 30 seconds ->Extension completion.

4°C: Storage.

2.12.4 ANALYSIS OF RESULTS.

After the run, the fluorescence of each tube at each cycle is graphed by the software, and a threshold is set between 20-25 cycles, where the PCR is seen to be in its exponential phase. The software then calculates the cycle at which the fluorescence in each tube crossed this threshold, which is termed the C_t. This C_t is then compared to the C_t of the housekeeping gene for the same cDNA sample, to give an indication of the relative level of expression of the Apo-E mRNA.

2.13 GENOTYPING FOR APO-E.

2.13.1 PCR OF THE APO-E GENE.

A simplified single stage PCR using 2 primers was used [Wenham et al 1991]. The primers amplify a 227 bp region of DNA that spans both Apo-E polymorphic sites. In the PCR 5µL of a 1 in 10 dilution of buffy coat or 5µl of DNA standard were added to the following:

Reaction buffer:	(10X)	2.5µl
DMSO:		2.5µl
$MgCl_2$:	(25mM)	2.5µl
dNTPs:	(10µM each)	1.0µl
Primer E23:	(25µM)	1.0µl
Primer E31	(25µM)	1.0µl
dH_2O:		8.5µl

A hot start PCR was used, where reagents were incubated for 5 minutes at 95°C and then at 80°C for 5 minutes during which time 1µL of diluted Taq DNA Polymerase (5000 U/mL) were added. Final reaction volume was 25µL. The thermocycler (Perkin Elmer) was programmed as follows:

94°C: 30 seconds ->Denaturation.]
65°C: 30 seconds ->Primer annealing.] 40 cycles.
74°C: 90 seconds ->Extension.]
74°C: 10 minutes ->Extension completion.
4°C: Storage

2.13.2 DIGESTION OF PCR PRODUCT.

3µL of diluted Cfo I restriction enzyme (Boehringer Mannheim, 1 in 15 dilution of 10 U/µl in 10X buffer) was added to each tube of amplified product and incubated at 37°C overnight in the thermocycler.

2.13.3 TBE-PAGE ELECTROPHORESIS.

5µL of loading buffer containing 30% glycerol and 0.25% bromophenol blue in TBE (0.045M Tris-borate, 0.001M EDTA, pH 8.0) was added to each tube of amplified PCR product. 15µl from each tube was loaded onto a 17.5% polyacrylamide gel. 1µL of a 100bp ladder (Boehringer Mannheim, 0.25 µg/µL) was also loaded with the same loading buffer. Electrophoresis was carried out at 100V for approximately 3 hours using TBE as running buffer. The gel was stained in Ethidium Bromide solution for 15 minutes, destained in distilled water, visualised using UV light and photographed.

There are 3 principal alleles of the Apo-E gene, ε2, ε3, and ε4. There are 6 possible genotypes (ε2/ε2, ε3/ε2, ε4/ε2, ε3/ε3, ε4/ε3 and ε4/ε4) which can be determined by visualising the combination of bands on the EtBr stained gel at 91, 81, 72, and 48 bp

.

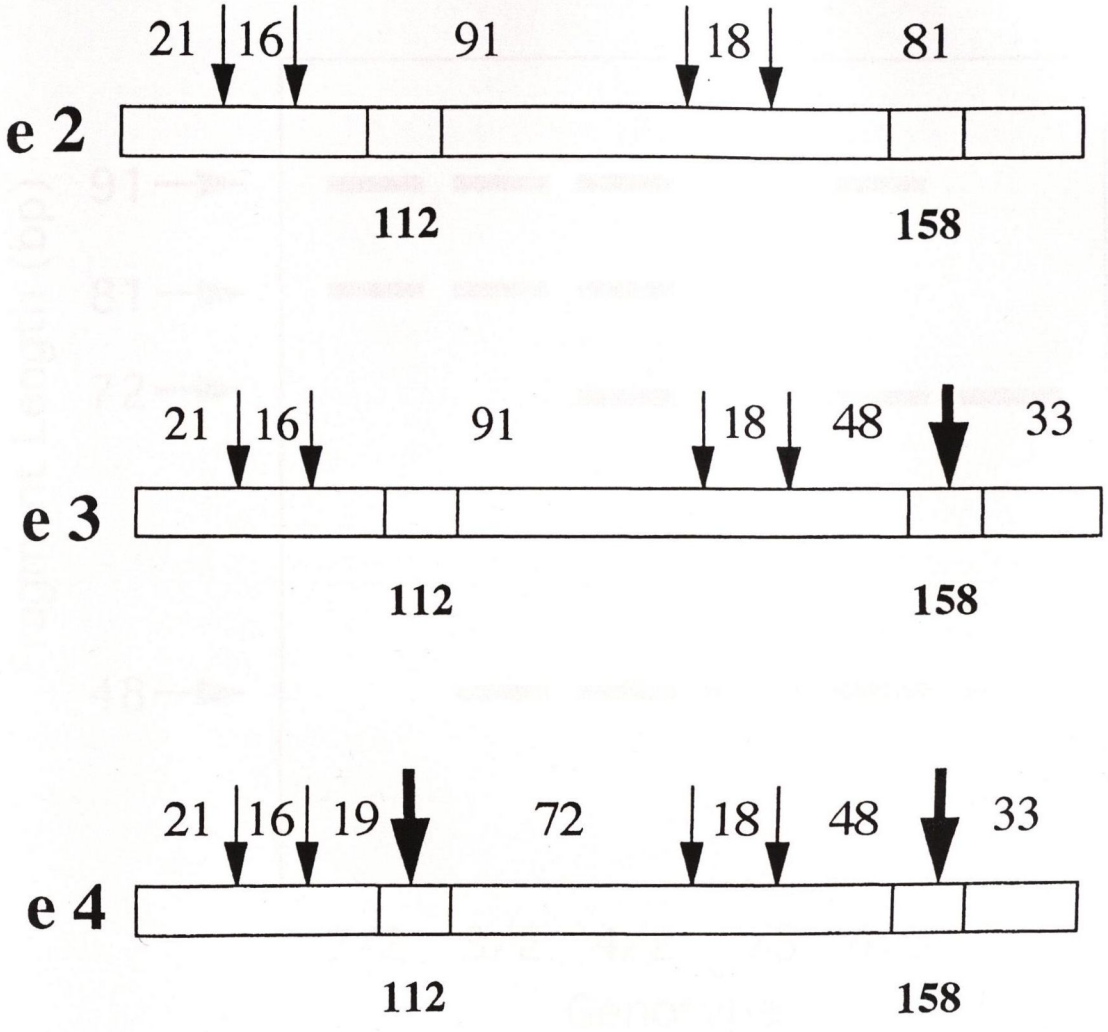

Figure 2.4: Cfo I restriction map of the major Apo-E alleles: *For the alleles ε2, ε 3 and ε 4, constant restriction sites are illustrated by the solid arrows and polymorphic sites by the bold arrows. The numbers indicate the size, in base pairs, of the restriction fragments formed.*

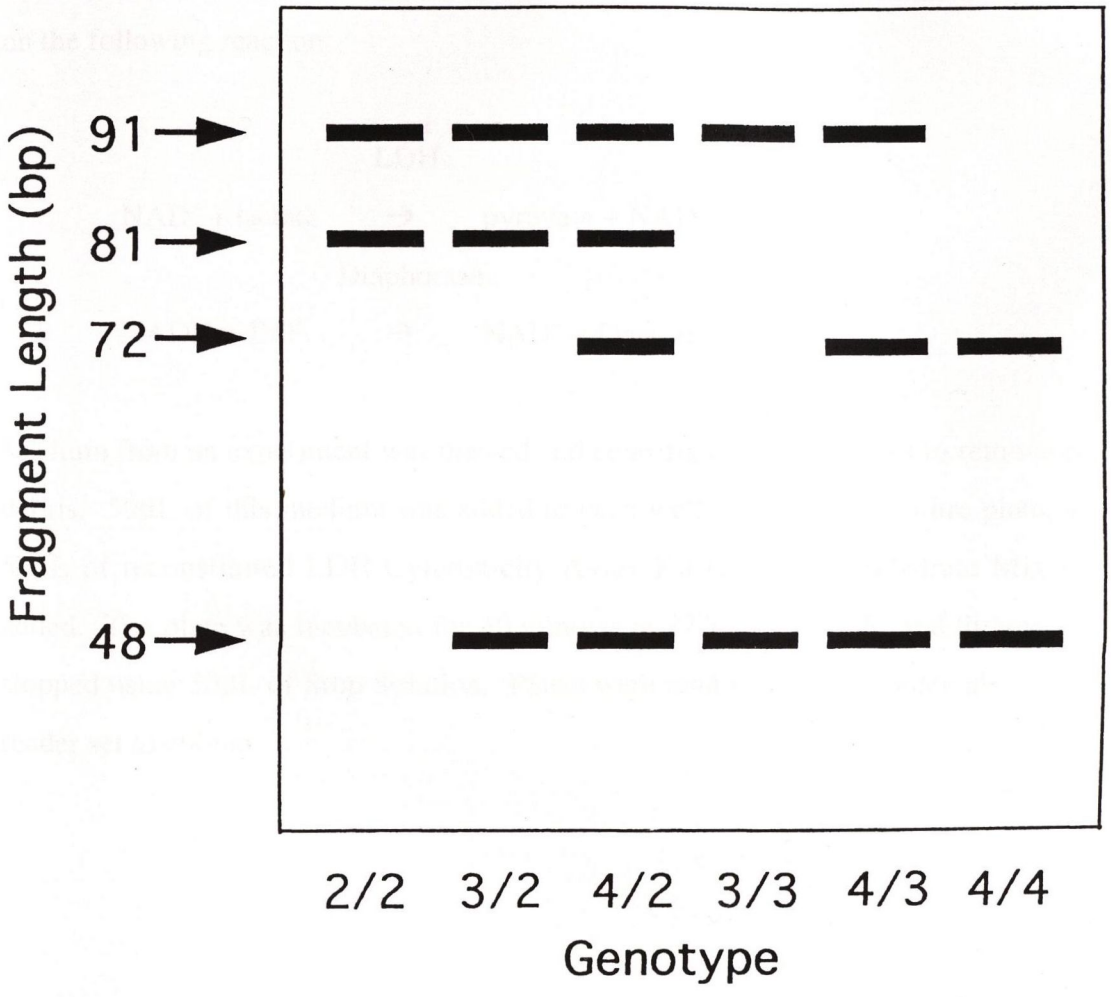

Figure 2.5: Schematic illustration of the Cfo I restriction pattern obtained from the six common Apo-E genotypes: *The size of the major DNA fragments is given in base pairs. Only the four largest fragments are shown here, and there may be some primer dimers at smaller weights.*

2.14 LDH CYTOTOXICITY ASSAY.

The detection method employed to assess cytotoxicity was based on the fact that when cells die by either necrosis or apoptosis, they lyse and release Lactate Dehydrogenase enzyme into the medium. The colorimetric change observed is based on the following reaction:

$$\text{NAD}^+ + \text{lactate} \xrightarrow{\text{LDH}} \text{pyruvate} + \text{NADH}$$

$$\text{NADH} + \text{INT} \xrightarrow{\text{Diaphorase}} \text{NAD}^+ + \text{formazan (red)}$$

Medium from an experiment was thawed and centrifuged for 5 minutes to remove cell debris. 50µL of this medium was added to each well of a clear microtitre plate, and 50µL of reconstituted LDH Cytotoxicity Assay Kit (Promega) Substrate Mix was added. The plate was incubated for 30 minutes at 37°C in the dark, and the reaction stopped using 50µL of Stop Solution. Plates were read in a 96 well plate absorbance reader set to 490nm.

CHAPTER 3

THE MODULATION OF APOLIPOPROTEIN-E mRNA AND PROTEIN IN ASTROCYTOMA CELL LINES BY CYTOKINES.

The aim of the work reported in this section was to set up a cell culture model reflecting the environment found at the astrocyte during an inflammatory MS attack. This was to be done using the transformed cell lines 1321N1 and C6. Apo-E mRNA, translated Apo-E protein and secreted Apo-E protein were all to be measured. Attempts would then be made to influence this production using a variety of inflammatory cytokines.

The results obtained will be presented under the following headings:

3.1 DIG labelled cDNA probe construction.

3.2 Estimation of RNA yield and quantity.

3.3 Digoxigenin labelled probing for Apo-E mRNA.

3.4 Modulation of Apo-E mRNA transcription in astrocytoma cells by inflammatory cytokines.

3.5 Western blotting for Apo-E in astrocytoma cells.

3.6 ^{35}S labelling and immunoprecipitation of Apo-E from 1321N1 cells.

3.7 LDH cytotoxicity assay on astrocytoma experiments.

3.1 DIG LABELLED cDNA PROBE CONSTRUCTION.

DIG labelled probes were used as an alternative to the traditional ^{32}P labelled type. Although slightly more difficult to optimise for signal and background, the probes are relatively non-toxic and reusable. If the DIG labelling process of cDNA fragments is allowed to proceed for 24hrs, there is a maximum amount of probe produced, about 5000ng in the final 20µL reaction mixture.

3.1.1 APO-E cDNA ISOLATION.

The gel pictured in 3.1.1 indicated that good plasmid preparations were obtained by phenol/chloroform extraction, and that the restriction digest had produced the required linearised plasmids. The linearised plasmid can be seen at approximately 3500bp. These could then be cut from the gel and used to prepare the cDNA probe. Several other bands are evident in this gel, and are most likely concatenated plasmids.

3.1.2 β-ACTIN cDNA ISOLATION.

Similarly the gel pictured in Figure 3.1.2 demonstrates that good β-actin plasmid preparations were obtained and that the restriction digest had excised the required cDNA insert. The cDNA insert can be seen at approximately 2000bp. These could then likewise be cut from the gel and used to prepare cDNA probes. Some smearing of the sample is evident at higher molecular weights, indicating multiple Hind III restriction sites on the E.Coli genome.

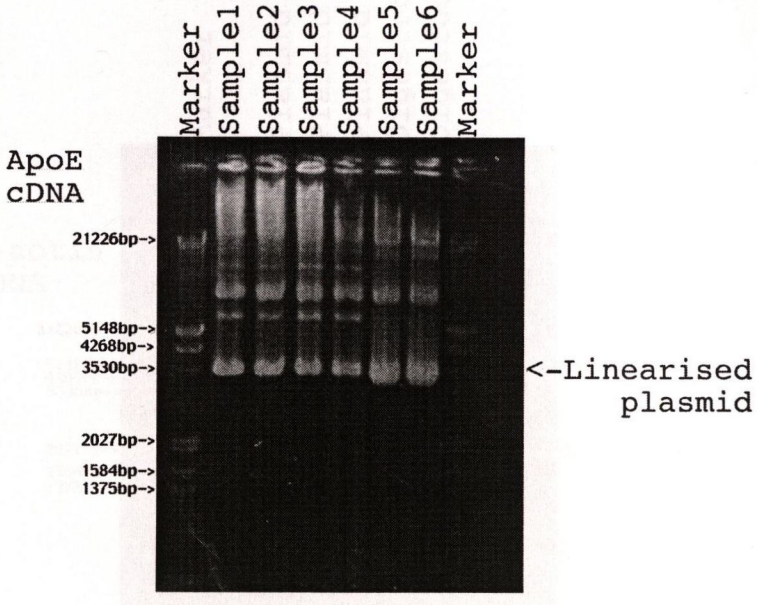

Figure 3.1.1: Endonuclease digested Apo-E cDNA containing plasmid electrophoresed in 1% agarose gel: *Apo-E cDNA containing plasmid, transfected into and grown in E.Coli, was isolated and digested with Bam H1 restriction endonuclease and electrophoresed in a 1% agarose TAE gel, as described in section 2.2.5. This gel was then stained with ethidium bromide and viewed under UV light.*

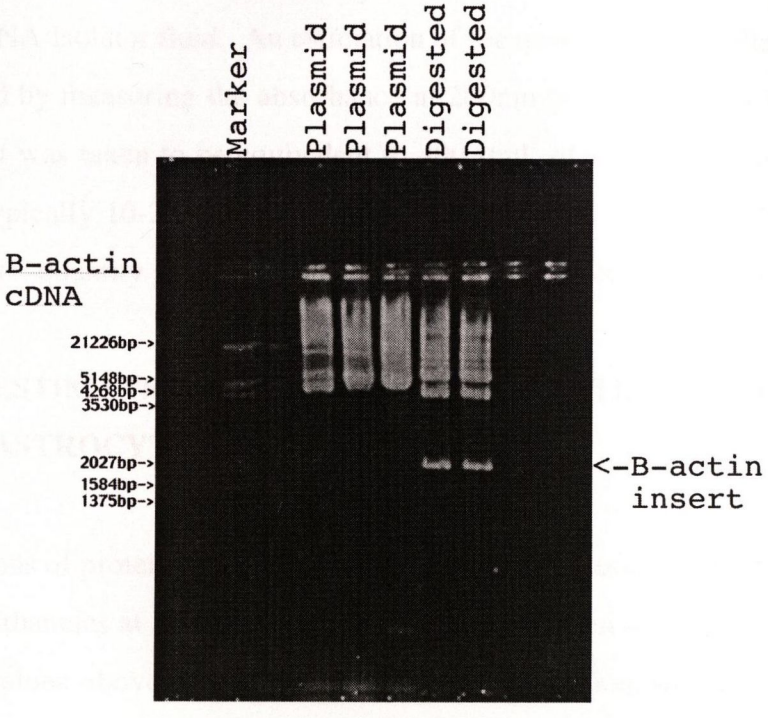

Figure 3.1.2: Endonuclease digested B-actin cDNA containing plasmid electrophoresed in 1% agarose gel: *β-actin cDNA containing plasmid, transfected into and grown in E.Coli, was isolated and digested with Hind III restriction endonuclease and electrophoresed in a 1% agarose TAE gel, as described in section 2.2.5. This gel was then stained with ethidium bromide and viewed under UV light.*

3.2 ESTIMATION OF RNA YIELD AND QUANTITY.

3.2.1 ESTIMATION OF THE YIELD OF TOTAL RNA FROM 1321N1 HUMAN AND C6 RAT ASTROCYTOMAS.

Total RNA was isolated from established and primary (see Section 4.3) cell lines using RNA Isolator fluid. An estimation of the quantity of RNA that was present was obtained by measuring the absorbance at 260nm of an aqueous solution of RNA. 1 A_{260} unit was taken to be equivalent to 40µg/mL of RNA. As can be seen in Table 3.2.1, typically 10-20µg of total RNA was extracted from a confluent well of a 24 well plate; quantity generally being greater for the faster growing C6 cell line.

3.2.2 ESTIMATION OF THE QUALITY OF EXTRACTED TOTAL ASTROCYTE RNA.

Indications of protein or DNA contamination were assessed by calculating the ratio of the absorbancies at A_{260}:A_{280}. Values of 2.0 were taken as indications of high quality RNA, values above 2.0 indicated DNA contamination and those below 2.0 were indicative of protein contamination. If protein or DNA was present, the RNA solution was either discarded or else re-extracted by addition of 200µL of RNA Isolator fluid followed by repetition of the RNA isolation steps. All RNA used in these experiments had A_{260}:A_{280} ratios within the range 1.8-2.0.

The quality of the mRNA in the solution of total RNA isolated was determined visually by a formaldehyde denaturing agarose gel. As can be seen from figure 3.2.2, a smear representing mRNA is present above and between the 28s and 18s ribosomal bands. RNA found close to the origin of the 1% (w/v) gel, or the absence of a smear on ethidium bromide staining, was taken to indicate that degradation of RNA had occurred.

Table 3.2.1: Estimation of the yield of total RNA from 1321N1 human and C6 rat astrocytomas.

Cell line	IFN-γ	ABS 260nm	ABS 280nm	260/280	µg/mL
1321N1	0	0.028	0.015	1.87	560
1321N1	0.001	0.032	0.018	1.78	640
1321N1	0.01	0.022	0.012	1.83	440
1321N1	0.1	0.031	0.016	1.94	620
1321N1	1	0.024	0.013	1.85	480
1321N1	10	0.022	0.011	2.00	440
C6	0	0.039	0.022	1.77	780
C6	0.001	0.041	0.022	1.86	820
C6	0.01	0.057	0.028	2.04	1140
C6	0.1	0.066	0.035	1.89	1320
C6	1	0.061	0.034	1.79	1220
C6	10	0.054	0.026	2.08	1080

RNA, isolated as described in section 2.7.2, was diluted 1:500 with DMPC treated H_2O and absorbances read at 260nm and 280nm. 260/280 ratios were calculated, and the 260nm reading used to calculate the concentration of RNA in the sample.

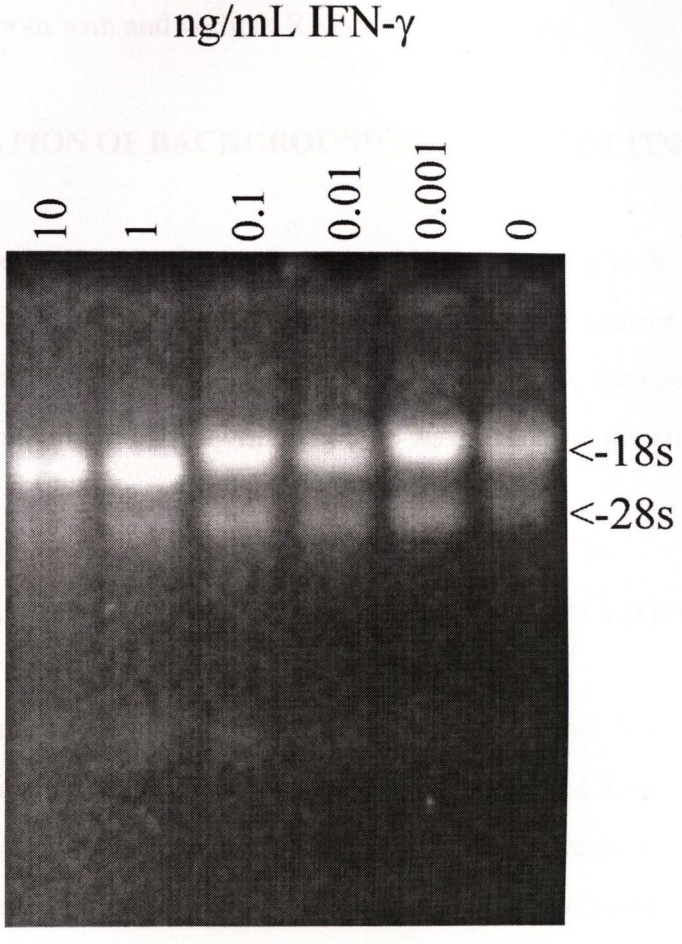

Figure 3.2.2: Estimation of the quality of extracted total RNA: *Total RNA was extracted from 1321N1 human astrocytoma cells and electrophoresed in a 1% agarose formaldehyde gel, as described in section 2.7.5. This gel was then stained with ethidium bromide and viewed under UV light.*

3.3 DIGOXIGENIN LABELLED PROBING FOR APO-E mRNA.

Optimisation of the DIG labelled probes is critical, as the technique is extremely sensitive and therefore prone to problems with background signal. To this end a series of test blots, both with and without RNA target, were used.

3.3.1 OPTIMISATION OF BACKGROUND FOR DIG BLOTTING.

The final DIG probe stock was taken to be about 5000ng of probe in 20μL. This was diluted with DIG Hybridisation Buffer to give a range of probe concentrations. The levels of background seen at over 10ng/mL (see Figure 3.3.1), for each probe, was unacceptable, so a maximum of 10ng/mL of probe was set for optimisation with RNA samples.

3.3.2 OPTIMISATION OF SIGNAL FROM QUANTIFIED TOTAL RNA.

This DIG slot blot, shown in Figure 3.3.2, indicates that the intensity of signal obtained from exposure to film is proportional to the amount of RNA applied to the blot. It also shows that at lower concentrations of probe there is only a very faint signal. Some contamination is evident at the edges of the blots, perhaps from a non-sterile scissors, but this does not affect the bands viewed. Final concentration of probes were decided on as: Apo-E = 5 ng/mL, β-actin =10 ng/mL. Later, after probing was further optimised through practice, these concentration were reduced to: Apo-E = 0.5 ng/mL, β-actin =1 ng/mL.

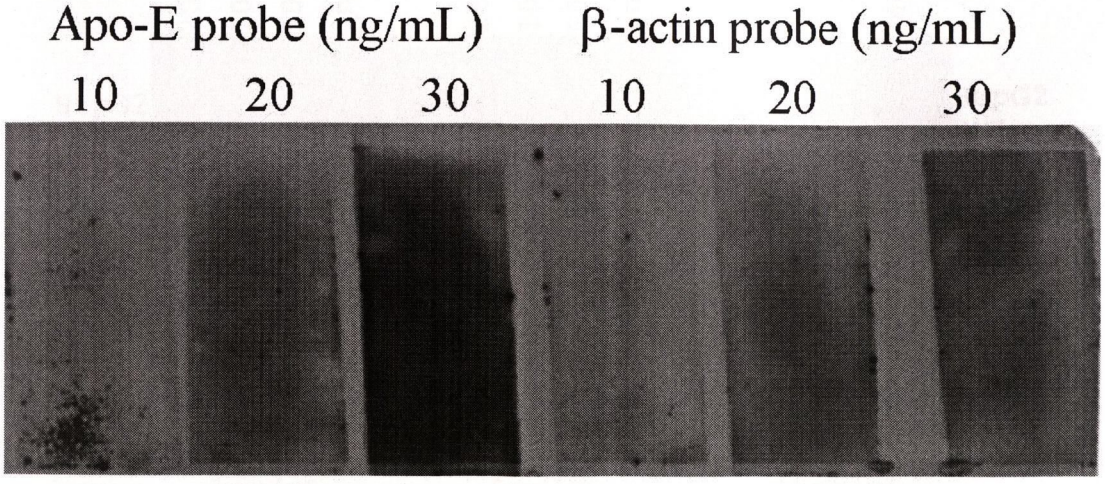

Figure 3.3.1: Optimisation of background for DIG blotting: *Various concentrations of the two DIG labelled probes to be used were hybridised with blank nylon membranes, as described in section 3.3.1. These blots were then visualised using CSPD substrate and exposure to film.*

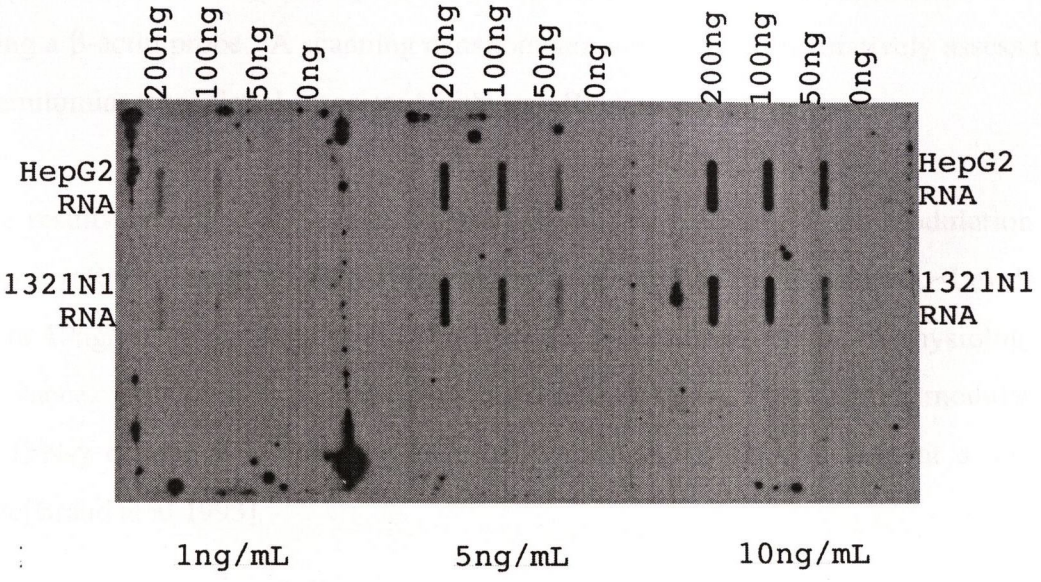

Figure 3.3.2: Optimisation of signal from quantified total RNA: *Varying amounts of total RNA isolated from 1321N1 and control HepG2 cell lines were applied to nylon membranes and probed for Apo-E coding RNA using varying concentrations of DIG labelled cDNA probe, as described in section 3.4.2.*

3.4 MODULATION OF APO-E mRNA TRANSCRIPTION IN ASTROCYTOMA CELLS BY INFLAMMATORY CYTOKINES.

1321N1 astrocytoma cells were grown to confluency, and treated with FCS free medium containing one of a number of the inflammatory cytokines known to be found in elevated levels during an MS relapse [Gironi et al 2000]. RNA isolated from these cells after 5 days was probed for Apo-E mRNA, and the results normalised using a β-actin probe. A scanning densitometer was used to quantitatively assess the chemiluminescent signal associated with each RNA sample.

The results presented in Figure 3.4a would indicate that there is no modulation of Apo-E mRNA transcription by IFN-γ at any concentration in the medium. Levels of up to 10ng/mL (equivalent to 100U/mL) could be considered to be of physiological relevance. This reflects findings in cultured macrophages, where Apo-E modulation by IFN-γ occurs at a post-transcriptional level and mRNA is kept at a steady state[Brand et al 1993].

The data shown in Figure 3.4b would indicate repression of Apo-E mRNA transcription by TNF-α. Expression falls by more than 50% in the presence of 1ng/mL. This reflects findings in cultured macrophages, where Apo-E modulation by TNF-α occurs at a transcriptional level, through control of gene expression at the genome or through accelerated degradation of Apo-E mRNA. Similarly, the data in Figure 3.4c would indicate strong repression of Apo-E mRNA transcription by IL-1α, through similar control mechanisms. The conclusions from these finding would be that under the influence of a number of the cytokines elevated during the MS relapse, astrocytes do not produce as much Apo-E, certainly not at the mRNA level.

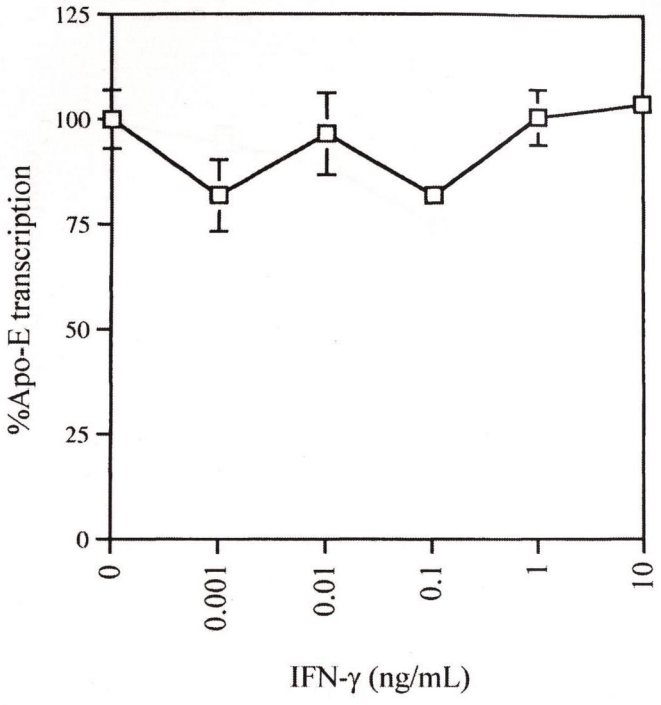

Figure 3.4a: Modulation of Apo-E mRNA from 1321N1 cells incubated with IFN-γ for 5 days: *Intensity of Apo-E mRNA signal was normalised against that of β-actin mRNA and plotted against IFN-γ concentration for a 5 day incubation of 1321N1 human astrocytoma cells. Graph is a mean of three experimental results, with standard deviation error bars.*

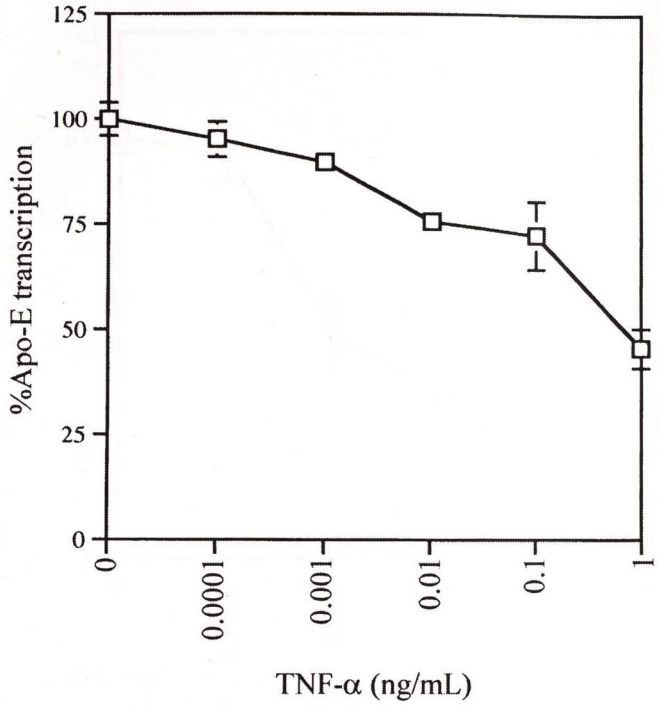

Figure 3.4b: Modulation of Apo-E mRNA from 1321N1 cells incubated with TNF-α for 5 days: *Intensity of Apo-E mRNA signal was normalised against that of β-actin mRNA and plotted against TNF-α concentration for a 5 day incubation of 1321N1 human astrocytoma cells. Graph is a mean of three experimental results, with standard deviation error bars.*

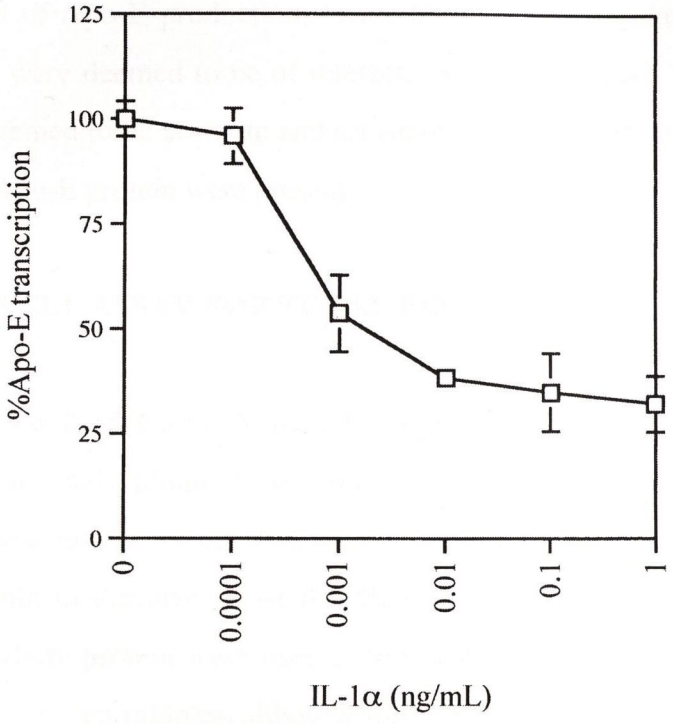

Figure 3.4c: Modulation of Apo-E mRNA from 1321N1 cells incubated with IL-1α for 5 days. *Intensity of Apo-E mRNA signal was normalised against that of β-actin mRNA and plotted against IL-1α concentration for a 5 day incubation of 1321N1 human astrocytoma cells. Graph is a mean of three experimental results, with standard deviation error bars.*

3.5 WESTERN BLOTTING FOR APO-E IN ASTROCYTOMA CELLS.

After assessing the modulation of mRNA levels of Apo-E in the astrocyte, the more physiologically important protein levels were investigated. The macrophage experiments conducted by Brand and Mazzone, 1993, would seem to indicate several levels of control of Apo-E production, hence both cellular translated and medium secreted protein were deemed to be of interest. Semi-quantitative Western blotting methods were deemed to be accurate and sensitive enough for this model, presuming fair amounts of Apo-E protein were present.

3.5.1 MARKWELL ASSAY FOR TOTAL PROTEIN.

It was decided to scale down the Markwell protein assay, from the commonly used 3mL or 5mL tube final volume, to use microtitre plates in order to maximise the amount of protein available for the Western blots. Samples were thawed and warmed to 37°C for 10min to dissolve all of the SDS before 10μL of cellular or 1μL of concentrated medium protein were used in the assay. A_{650} readings were normally rather consistent between samples, although sometimes there was a small drop in total cellular protein evident at high levels of IFN-γ.

3.5.2 TEST BLOTTING VERSUS HUMAN PLASMA.

In order to test the sensitivity of the Dako anti-Apo-E antibody, human plasma from a control subject was obtained, and diluted to give a range between 1μL and 0μl. These samples were examined by Western blotting using a range of antibody dilutions, followed by 4-chloro-1-napthol staining. It appears that all the concentrations of antibody examined gave a good signal, and that a plasma volume of between 0.1μL or 0.01μL would give a good standard signal without overloading the gels with total protein.

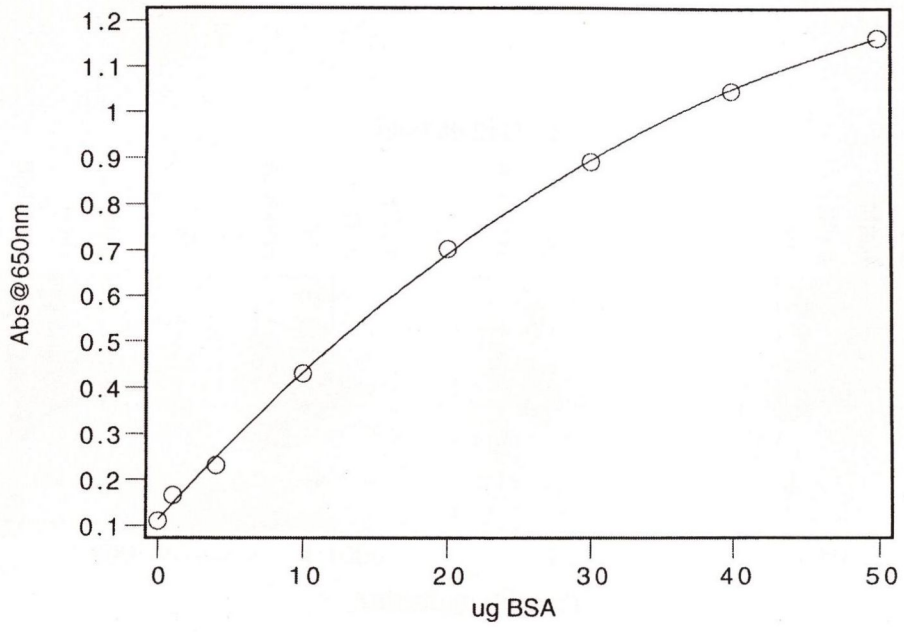

Figure 3.5.1: Markwell assay for total protein: *Various amounts of BSA protein were used in the Markwell assay described in section 2.10. The absorbance of each sample at 650nm in a spectrophotometer was noted, and used to construct a graph. The protein concentration of samples could then be determined by extrapolation from this graph.*

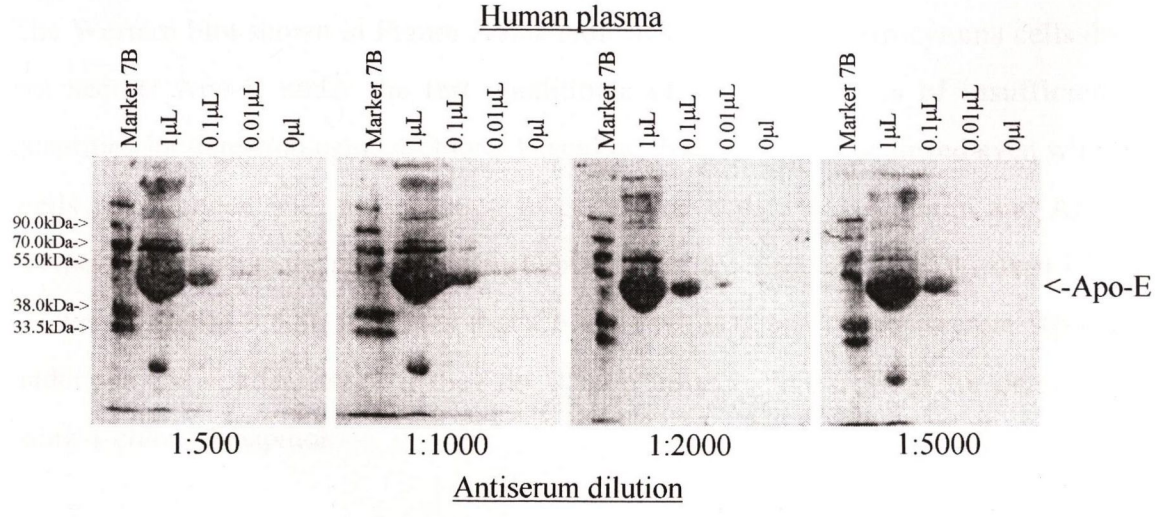

Figure 3.5.2: Test blotting versus human plasma: *Varying amounts of human plasma were loaded into a 15% PAGE SDS gel, electrophoresed, and transferred to nitrocellulose. This blot was then probed with varying concentrations of anti-Apo-E antibody and stained with 4-chloro-1-napthol, as described in section 2.11.*

3.5.3 4-CHLORO-1-NAPTHOL STAINING OF WESTERN BLOTS.

It was expected that there would be far larger amounts of Apo-E protein accumulated in the medium than in the cell extracts, hence to begin with, the concentrated medium from 5 day experimental runs was examined by Western blotting. A range of cytokines were generally used in these experiments, and staining was done with 4-chloro-1-naphthol, which was shown to be sensitive to as little as 3ng of Apo-E (0.1μL of plasma at roughly 0.03g/L, the common physiological concentration).

The Western blot shown in Figure 3.5.3a indicates that 1321N1 astrocytoma cells do not secrete Apo-E under the test conditions, or if they do, it is of insufficient quantities for detection using 4-chloro-1-napthol. No bands were achieved even when wells were loaded with up to 100μg of concentrated protein. Albumin and Apo-E/Apo-AII dimer were also noted in human plasma. Similarly, the Western blot shown in Figure 3.5.3b indicates that C6 astrocytoma cells do not secrete Apo-E under the test conditions, or if they do, it is in insufficient quantities for detection using 4-chloro-1-napthol.

After these blots, it was concluded that the 1321N1 and C6 cell lines do not secrete sufficient Apo-E for detection with the 4-chloro-1-napthol method. Another much more sensitive method was adopted, the chemiluminescent substrate, for all further experiments.

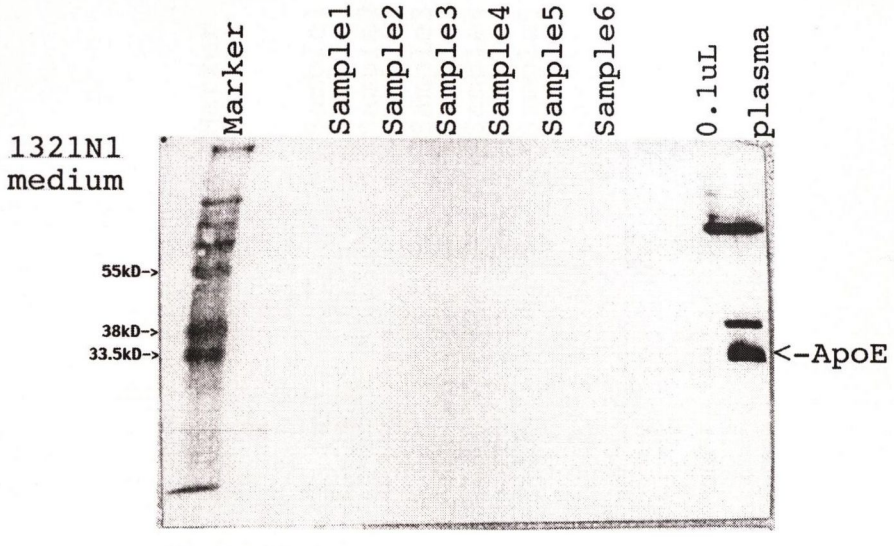

Figure 3.5.3a: 4-chloro-1-napthol staining of Western blot for Apo-E against 1321N1 secreted protein: *Concentrated secreted protein was prepared from a 5 day culture of 1321N1 cells and 50µg loaded into a 15% PAGE SDS gel, electrophoresed, and transferred to nitrocellulose. This blot was then probed with anti-Apo-E antibody and stained with 4-chloro-1-napthol, as described in section 2.11.*

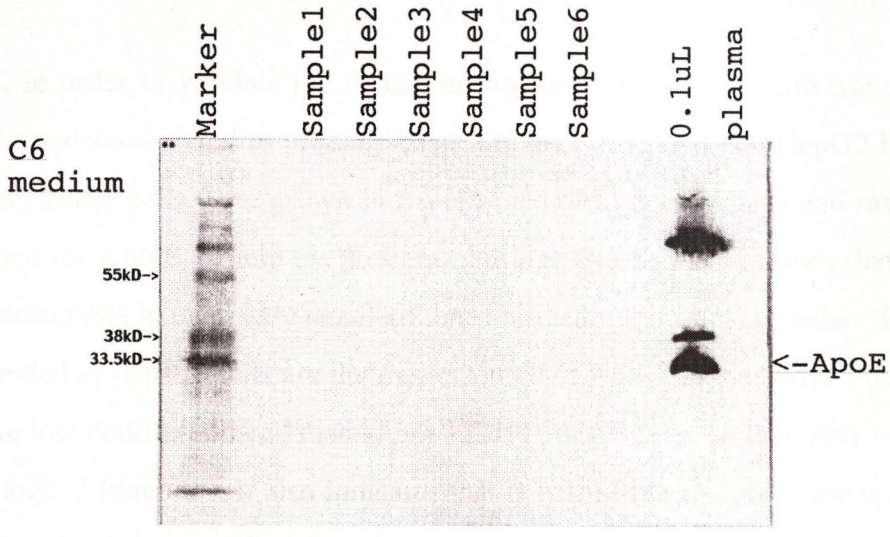

Figure 3.5.3b: 4-chloro-1-napthol staining of Western blot for Apo-E against C6 secreted protein: *Concentrated secreted protein was prepared from a 5 day culture of C6 cells and 50µg loaded into a 15% PAGE SDS gel, electrophoresed, and transferred to nitrocellulose. This blot was then probed with anti-Apo-E antibody and stained with 4-chloro-1-napthol, as described in section 2.11.*

3.5.4 CHEMILUMINESCENT STAINING OF WESTERN BLOTS.

Following the failure to detect Apo-E protein, it was thought that perhaps the 4-chloro-1-napthol was of insufficient sensitivity to measure the Apo-E secretions from these cell lines. It was also decided to extend the range of the blots to also cover cellular extracts. Accumulation of Apo-E in the cell and failure to secrete may be an explanation for the results seen in Section 3.4.

Firstly, in order to validate the detection process, it was decided to use a cell line previously demonstrated to produce large amounts of Apo-E, the HepG2 hepatocyte cell line. These cells were grown in the absence of FCS for 5 days and raw medium examined for Apo-E protein by Western blotting. Figure 3.5.4a shows that Apo-E is easily detectable in even very small volumes of medium from these cells. This would be expected as hepatic cells are the major source of Apo-E in the body. Also, HepG2 cells are less dedifferentiated than either 1321N1 or C6 cells, so they may retain more physiological function. It also indicates that 0.1μL of plasma gives too much signal with chemiluminescent substrate.

100μg of secreted protein from a 5 day, FCS free, C6 glioma culture was loaded into each well of a gel and examined by Western blotting. Figure 3.5.4b indicates that C6 astrocytoma cells do not secrete Apo-E under the test conditions, or if they do, it is in insufficient quantities for detection using chemiluminescent staining. Albumin cross-reactivity and Apo-E/Apo-AII dimer were also noted in human plasma.

The Western blot shown in Figure 3.5.4c indicates that 1321N1 astrocytoma cells also do not translate Apo-E under the test conditions. No bands were achieved with this cell line either when wells were loaded with up to 100μg of cellular protein. Some background signal is seen due to the excessive exposure time of 2 hours. Also it was noted that 0.01μL of human plasma was easily sufficient for use as a standard with this technique.

Figure 3.5.4d indicates that 1321N1 astrocytoma cells do not secrete Apo-E under the test conditions, despite maximum loading of protein into the gel and sufficient exposure times with the chemiluminescent substrate to start to produce background.

It was concluded from these Western blots, which are sensitive to only a few nanograms of Apo-E, were most likely correct and there was no protein being translated. This may have been due to one of two factors. There may be insufficient cholesterol and lipid in the FCS free environment of the experiment to allow accumulation of Apo-E protein. In this case, Apo-E mRNA and protein is rapidly degraded and cannot be detected.

Another scenario is that the fast growing, highly de-differentiated 1321N1 and C6 cell lines have lost the ability to translate Apo-E protein. There would obviously be very little use for Apo-E production by a cell line that receives no benefit from such a protein. Investigations into Apo-E production in the brain are normally conducted using primary animal cells, and the highly complex lipid secreting machinery involved may easily be lost during mutation into an established cell line.

From these experiments it is concluded that Apo-E accumulation in cellular extracts or secreted proteins is not detected by a sensitive technique that can detect the amount of Apo-E in 0.01μL of human plasma, typically of the order of 50ng.

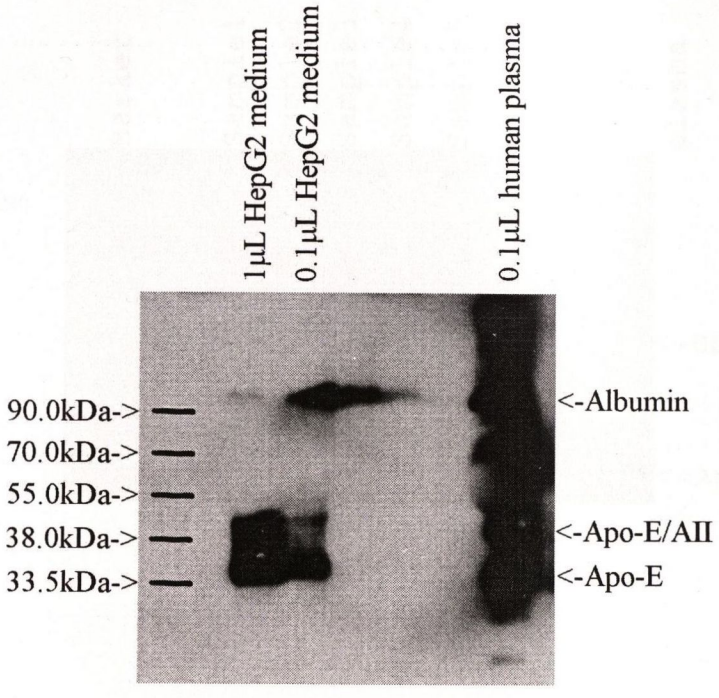

Figure 3.5.4a: Chemiluminescent staining of Western blot for Apo-E against HepG2 secreted protein: *Medium from a 5 day culture of HepG2 liver cells was loaded into a 15% PAGE SDS gel, electrophoresed, and transferred to nitrocellulose. This blot was then probed with anti-Apo-E antibody and visualised using a chemiluminescent substrate, as described in section 2.11.*

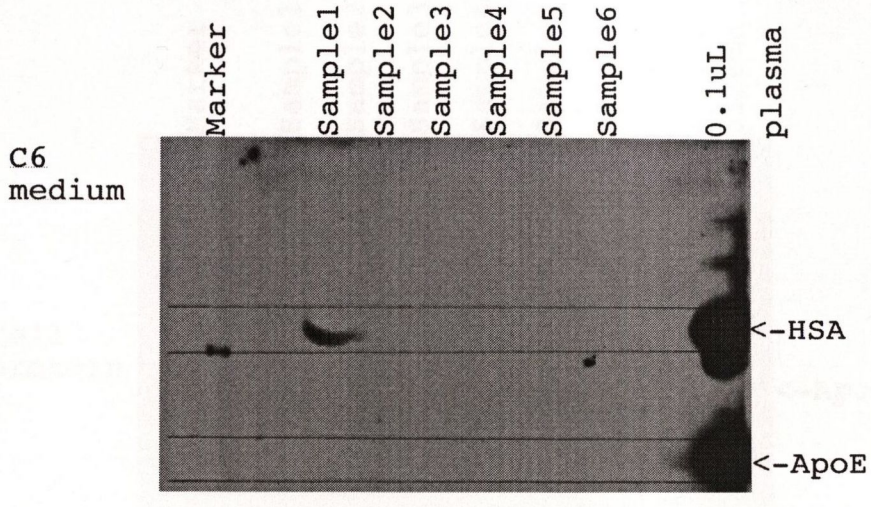

Figure 3.5.4b: Chemiluminescent staining of Western blot for Apo-E against C6 secreted protein: *Concentrated secreted protein was prepared from a 5 day culture of C6 cells and 50μg loaded into a 15% PAGE SDS gel, electrophoresed, and transferred to nitrocellulose. This blot was then probed with anti-Apo-E antibody and visualised using a chemiluminescent substrate, as described in section 2.11.*

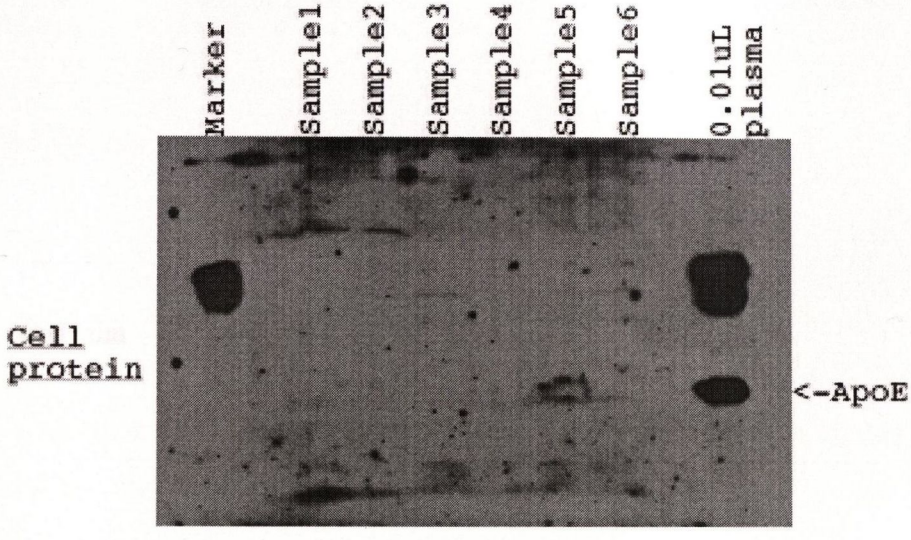

Figure 3.5.4c: Chemiluminescent staining of Western blot for Apo-E against 1321N1 cellular protein: *Total cellular protein was extracted from a 5 day culture of 1321N1 cells and 50μg loaded into a 15% PAGE SDS gel, electrophoresed, and transferred to nitrocellulose. This blot was then probed with anti-Apo-E antibody and visualised using a chemiluminescent substrate, as described in section 2.11.*

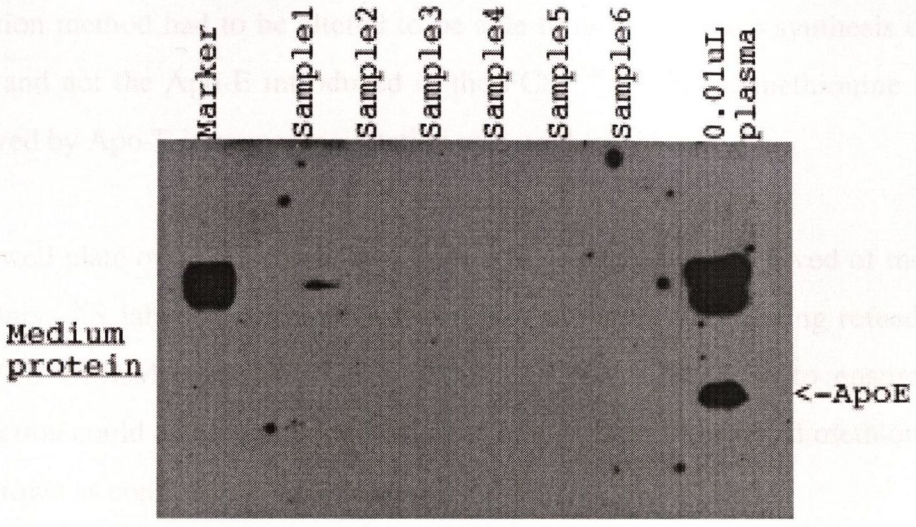

Figure 3.5.4d: Chemiluminescent staining of Western blot for Apo-E against 1321N1 secreted protein: *Concentrated secreted protein was prepared from a 5 day culture of 1321N1 cells and 50µg loaded into a 15% PAGE SDS gel, electrophoresed, and transferred to nitrocellulose. This blot was then probed with anti-Apo-E antibody and visualised using a chemiluminescent substrate, as described in section 2.11.*

3.6 ^{35}S LABELLING AND IMMUNOPRECIPITATION OF APO-E FROM 1321N1 CELLS.

It was decided to conduct an experiment to assess whether the absence of lipid and cholesterol in the experimental conditions were having an adverse effect on Apo-E production in 1321N1 and C6 cells. In order to introduce FCS into the medium, the detection method had to be altered to be able to assess de-novo synthesis of Apo-E only, and not the Apo-E introduced in the FCS. ^{35}S labelled methionine labelling followed by Apo-E immunoprecipitation was used for this.

A 24 well plate of 1321N1 cells was grown to confluency and starved of methionine for 24hrs. ^{35}S labelled methionine was added to the medium during refeeding with 10%FCS DMEM medium. Cold methionine was also added to ensure protein production could continue to completion. Cells with no ^{35}S labelled methionine were also grown as controls.

3.6.1 LIQUID SCINTILLATION COUNTING OF TOTAL CELLULAR UPTAKE OF ^{35}S LABELLED METHIONINE.

After taking a range of samples to ensure sufficient radioactivity for detection, resuspended cellular protein samples were mixed with scintillation fluid and DPMs measured in a scintillation counter. As can be seen from Figure 3.6.1, the amount of ^{35}S incorporated into the cellular protein was linear with time. This meant that the cells were alive throughout the timecourse, as they were taking up ^{35}S labelled methionine for the duration.

3.6.2 COOMASSIE STAINING OF TOTAL CELLULAR AND MEDIUM PROTEIN.

After immunoprecipitation of Apo-E (see section 2.6.4) was conducted, cellular extracts and raw medium were electrophoresed (see section 2.11) in an SDS-PAGE

gel and examined by staining with Coomassie blue. As can be seen from Figure 3.6.2, there is a fairly constant amount of protein in the cellular extract over the timecourse, indicating that the cells were confluent at the outset and the total cellular mass had changed little. The large bands visible at around 60kDa, correspond to albumin (the heavier of the two bands at 66kDa), and to the heavy chain of the antibody used to immunoprecipitate the Apo-E from the solutions (the lighter chain at 55kDa).

3.6.3 COOMASSIE STAINING OF APO-E IMMUNOPRECIPITATED PROTEIN.

At this stage, the immunoprecipitated samples were simply a few microlitres of Protein-A Sepharose sludge, to which were added 10μL of SDS-Page loading buffer. Samples were electrophoresed (see section 2.11) in an SDS-PAGE gel and examined by staining with Coomassie blue. As can be seen in Figure 3.6.3, the total amount of protein present in each sample appeared to be roughly similar, although the vast majority of this was accounted for by the heavy and light chains of the antibodies used in the immunoprecipitation.

3.6.4 PPO SCINTILLATION OF TOTAL EXTRACTED PROTEIN IN SDS-PAGE GEL.

After staining with Coomassie, the post-immunoprecipitation sample gel was soaked in DMSO, followed by PPO scintillant, and then dried onto blotting paper (see section 2.6.7). Seven stacked films were placed on top of this gel in an autoradiographic cassette, and the furthest film from the blot removed for development each day. Only on the last 2 days was any signal registered, and as can be seen in the results presented in Figure 3.6.4, there are quite a number of proteins ^{35}S labelled, the intensity of signal from each increasing with time. Since the total cellular extract appeared to change very little with time, it can be assumed there is a gradual accumulation of labelled protein to replace normal protein, rather than any extra growth of cells.

Unfortunately, no bands at all were observed in the medium samples. This may be due to an insufficiently hot radionucleotide, or perhaps not enough of it was used. Normally experiments examining lipoprotein production use the same radioactive counts used here (200μCi/mL), so perhaps it is a characteristic of the 1321N1 cell line that very little protein is secreted.

3.6.5 PPO SCINTILLATION OF APO-E IMMUNOPRECIPITATED PROTEIN IN SDS-PAGE GEL.

Similarly to the post-immunoprecipitation sample gel, the Coomassie stained immunoprecipitant gel was soaked in DMSO, followed by PPO scintillant, and then dried onto blotting paper (see section 2.6.7). This time only on the 7th day was any signal registered, and as can be seen in Figure 3.6.5, several ^{35}S labelled proteins are visible, the intensity of signal from each increasing with time. There appears to be some cross-reactivity between the Apo-E antibody and other cellular proteins during immunoprecipitation. A very faint band is visible just below the 33.5kDa marker which may, in fact be Apo-E cellular protein, but even if it is, it is being translated in such low amounts as to render the 1321N1 cell line virtually useless as a model for Apo-E production.

These experiments essentially establish that immunodetectible Apo-E is not detected in 1321N1 or C6 cells with chloronapthol staining, chemiluminescent staining, or with ^{35}S methionine labelling methods. It was decided then to assess whether any of the results observed with the astrocytoma cells could be attributed to cytotoxic effects.

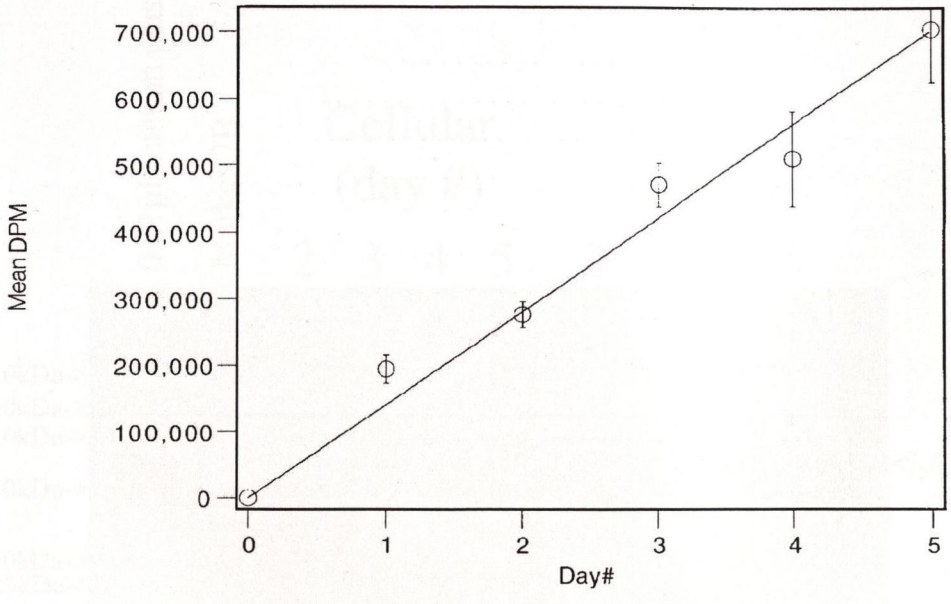

Figure 3.6.1: Liquid scintillation counting of total cellular uptake of ^{35}S labelled methionine: *17mm confluent wells of 1321N1 cells were incubated with 200mL of 200mCi/mL of ^{35}S labelled methionine for a 5 day timecourse, and 10μL of total cellular extract were analysed in a scintillation counter (see section 2.6). Results shown are a mean of three samples, with standard deviation error bars.*

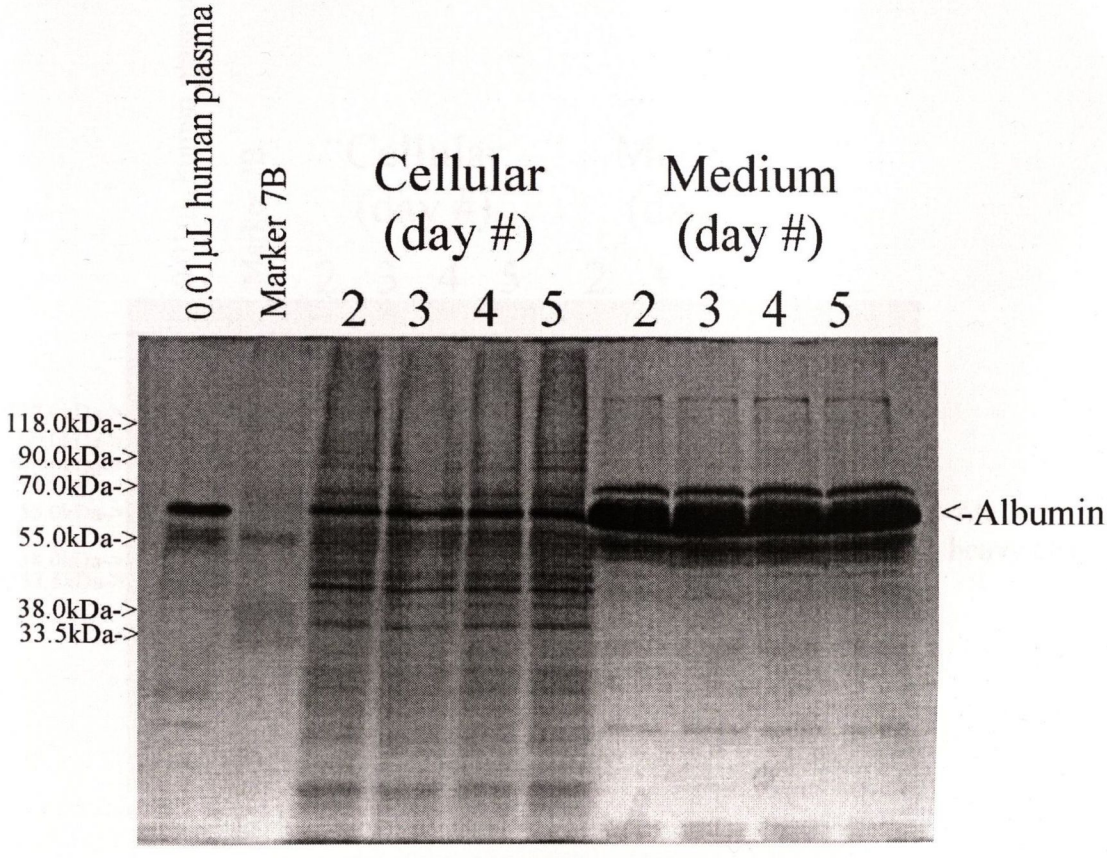

Figure 3.6.2: Coomassie staining of total cellular protein: *Post-immunoprecipitation cellular and medium samples (10µL each) from ^{35}S labelled 1321N1 cells were electrophoresed in a 15% SDS-PAGE gel and stained with Coomassie blue for total protein (see section 2.6).*

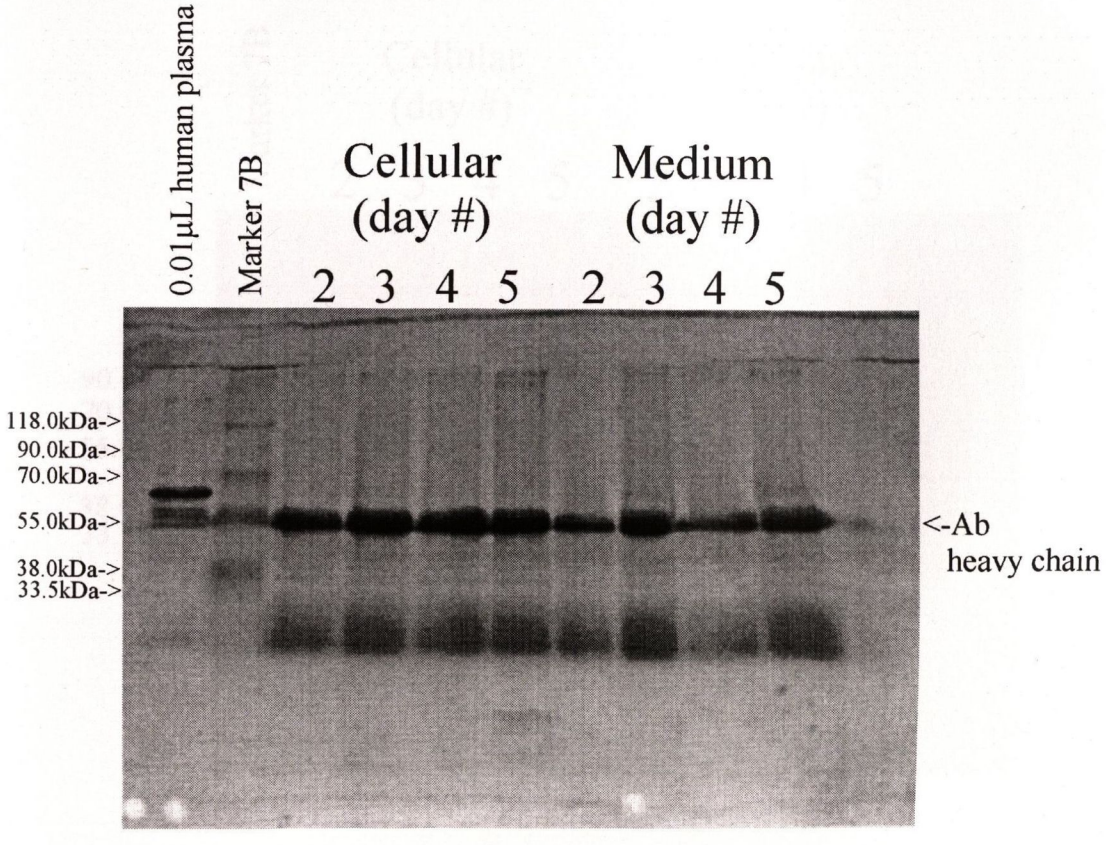

Figure 3.6.3: Coomassie staining of Apo-E immunoprecipitated protein: *Total immunoprecipitated cellular and medium samples from ^{35}S labelled 1321N1 cells were electrophoresed in a 15% SDS-PAGE gel and stained with Coomassie blue for total protein (see section 2.6).*

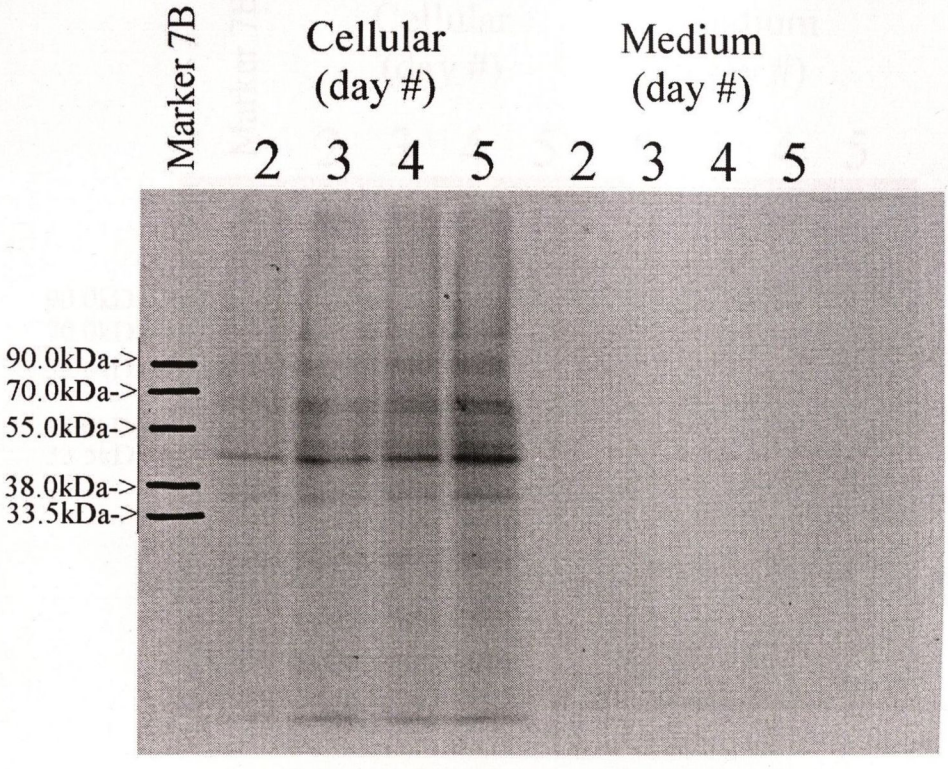

Figure 3.6.4: PPO scintillation of total extracted protein in SDS-PAGE gel: *After electrophoresis and staining for total protein, post-immunoprecipitation cellular and medium samples from ^{35}S labelled 1321N1 cells were dehydrated with DMSO and soaked in PPO scintillant. Dried gels were then exposed to film and developed after 7 days (see section 2.6).*

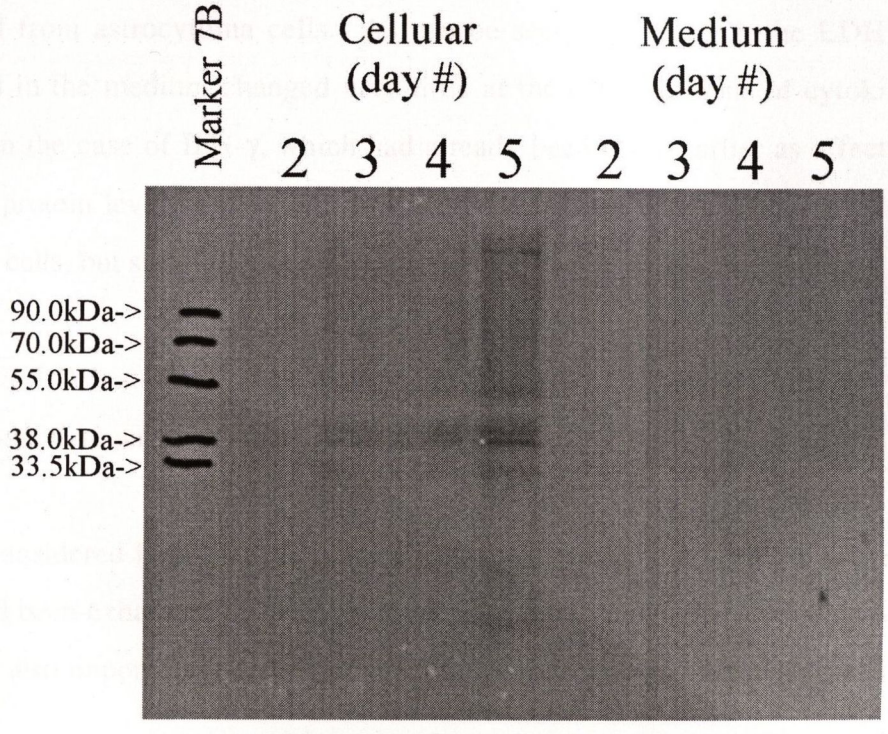

Figure 3.6.5: PPO scintillation of Apo-E immunoprecipitated protein in SDS-PAGE gel: *After electrophoresis and staining for total protein, Apo-E immunoprecipitated cellular and medium samples from ^{35}S labelled 1321N1 cells were dehydrated with DMSO and soaked in PPO scintillant. Dried gels were then exposed to film and developed after 7 days (see section 2.6).*

3.7 LDH CYTOTOXICITY ASSAY ON ASTROCYTOMA EXPERIMENTS.

Substances such as IFN-γ and TNF-α are known to be toxic to most cell types when present at high concentration. Therefore it was necessary to assess whether cytotoxicity was playing a role in the downregulation of Apo-E mRNA signal detected from astrocytoma cells. As can be seen in Table 3.7, the LDH activity detected in the medium changed very little at the concentrations of cytokine used, except in the case of IFN-γ, which had already been noted earlier as affecting total cellular protein levels somewhat. It was concluded that IFN-γ is somewhat toxic to 1321N1 cells, but still does not affect the Apo-E:β-actin mRNA ratios.

When an LDH standard was examined, 50µl of LDH at 3.3U/mL (= 0.165U) corresponded to an absorbance of 0.146 at 490nm.

It was considered here that the possibilities for investigation with the astrocyte cell lines had been exhausted. Therefore it was decided to move on to the monocyte cell, which is also important in Apo-E production, as well as being associated with the MS relapse.

Table 3.7: LDH cytotoxicity assay on 1321N1 experiments

IFN-γ (ng/mL)	10	1	0.1	0.01	0.001	0
Mean Abs @490nm	0.977	0.820	0.756	0.652	0.713	0.726
SD	0.034	0.052	0.056	0.022	0.014	0.080

TNF-α (ng/mL)	1	0.1	0.01	0.001	0.0001	0
Mean Abs @490nm	0.713	0.723	0.762	0.753	0.664	0.734
SD	0.043	0.079	0.029	0.006	0.032	0.046

IL-1α (ng/mL)	1	0.1	0.01	0.001	0.0001	0
Mean Abs @490nm	0.746	0.723	0.762	0.748	0.702	0.719
SD	0.089	0.079	0.029	0.080	0.083	0.083

50μL of thawed, cell free, medium was used in conjunction with the Cytotox LDH assay kit, and absorbancies read at 490nm. Higher readings indicate more cytotoxicity as LDH is released from cells.

CHAPTER 4

THE MODULATION OF APOLIPOPROTEIN-E mRNA AND PROTEIN IN PRIMARY AND TRANSFORMED HUMAN MONOCYTES BY CYTOKINES.

The aim of the work reported in this section was to examine the production of Apo-E by the macrophage in the MS environment. The production of Apo-E protein by the infiltrating macrophage is believed to be crucial to the recycling of lipids in the regenerating neuron. A model was to be constructed using the THP-1 transformed monocyte cell line, and later attempts would be made to reproduce this work in primary monocytes obtained from donor blood. Monocytes produce very little Apo-E, but during an inflammatory attack are activated, and differentiate to become adherent macrophages, which travel and attach to the site of inflammation. This activation is reproduced in cell culture using phorbol 12-myristate 13-acetate (PMA).

The results obtained were presented under the following headings:

4.1 Detection of Apo-E protein production in a THP-1 monocyte cell line by Western blotting.*

4.2 Detection of Apo-E protein production in primary monocyte cells by Western blotting.*

4.3 Detection of Apo-E mRNA production in a THP-1 monocyte cell line by RT-PCR.

4.4 Detection of Apo-E mRNA production in primary monocyte cells by RT-PCR.

4.5 LDH cytotoxicity assay on monocyte experiments.

(*Note: Absolute values for most of the Western blots in Sections 4.1 and 4.2 are available in Appendix 6 in the form of scanning densitometry data, and expressed in percentage form in Appendix 7).

4.1 DETECTION OF APO-E PROTEIN PRODUCTION IN A THP-1 MONOCYTE CELL LINE BY WESTERN BLOTTING.

In order to test that the THP-1 cells had retained functionality, 24mL of the THP-1 cell line received were activated with 10^{-7}M PMA and used to seed a 24-well plate at 1mL per well. Medium samples were taken and concentrated 20 fold on each day of a 5 day timecourse, and examined for Apo-E protein by SDS-PAGE followed by Western blotting. As the results presented in Figure 4.1 show, the THP-1 cells translate and secrete good amounts of Apo-E protein. Also visible in the secreted protein are lower molecular weight degradation products of the protein, which occur due to the instability of Apo-E. Since the intensity of the Apo-E bands seen in the medium increases with time, and is far greater than the intensity of the breakdown products, it would be reasonable to assume that there is accumulation of protein, rather than degradation and fresh secretion.

4.1.1 MODULATION OF APO-E TRANSLATION IN THP-1 CELLS BY CYTOKINES.

Since the THP-1 cell line was shown to be a good model for Apo-E translation and secretion, it was decided to introduce to the cells some of the cytokines found in the MS environment, namely the inflammatory cytokines, and IFN-β, which may be present when patients are receiving therapy. Firstly, resuspended cellular protein was analysed from THP-1 cells incubated in the presence of inflammatory cytokines in order to examine the control that they may have at a translational stage. As can be seen in Figure 4.1.1a, IFN-γ appears to adversely affect Apo-E cellular levels, even up to 6 days after addition to the cell culture. This repression occurs at the same concentrations which were shown to be non-inhibitory to Apo-E mRNA transcription in astrocytes (see section 3.4).

Neither TNF-α or IL-1α, which were both shown to affect astrocyte Apo-E mRNA, were seen to have any overly significant affect on Apo-E protein levels in THP-1 cells

after 5 days of incubation (Table 4.1.1b and 4.1.1c respectively). At 10^{-1}ng/mL of TNF-α there appears to be a more noticeable drop in Apo-E levels, as there was at 10^{-4}ng/mLof IL-1α and 10^{-7}ng/mL of TGF-β. The importance of these results may be in some way related to the non-dose dependent repression seen later in primary monocytes with IFN-β and IFN-γ. The influence of TGF-β was examined, but had to be kept at an extremely low concentration due to cytotoxic effects on the cells. No influence was observed with TGF-β either (see Table 4.1.1d).

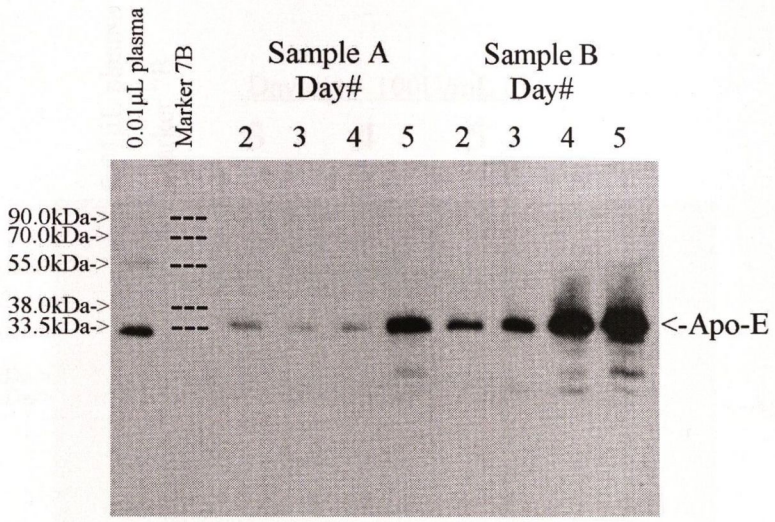

Figure 4.1: Chemiluminescent staining of Western blot for Apo-E against THP-1 secreted protein: *Concentrated secreted protein was prepared from each day of a 5 day culture of THP-1 cells and 50µg loaded into a 15% PAGE SDS gel, electrophoresed, and transferred to nitrocellulose. This blot was then probed with anti-Apo-E antibody and visualised using a chemiluminescent substrate, as described in section 2.11.*

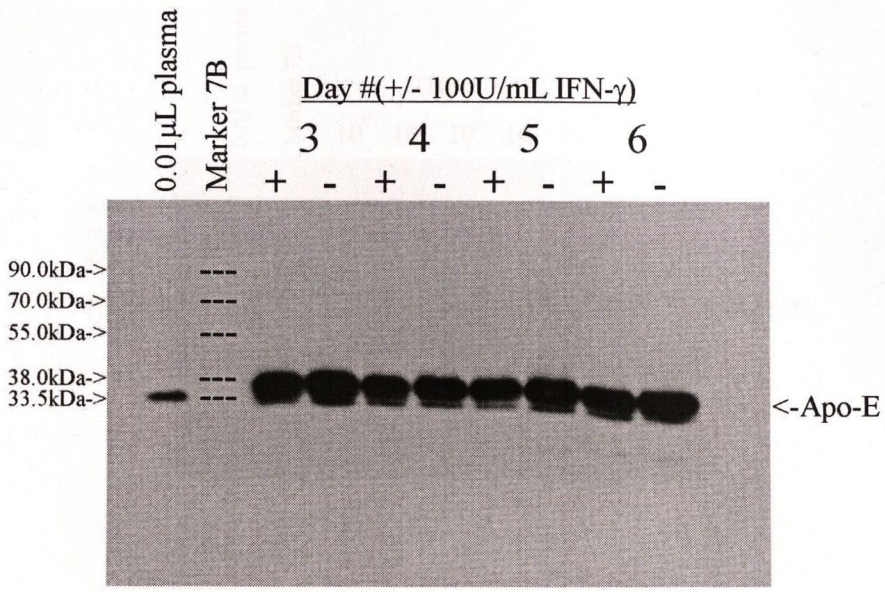

Figure 4.1.1a: Chemiluminescent staining of Western blot for Apo-E against cellular protein from IFN-γtreated THP-1 cells: *Total cellular protein was extracted on each day of a 6 day culture of THP-1 cells and 10µg loaded into a 15% PAGE SDS gel, electrophoresed, and transferred to nitrocellulose. This blot was then probed with anti-Apo-E antibody and visualised using a chemiluminescent substrate, as described in section 2.11.*

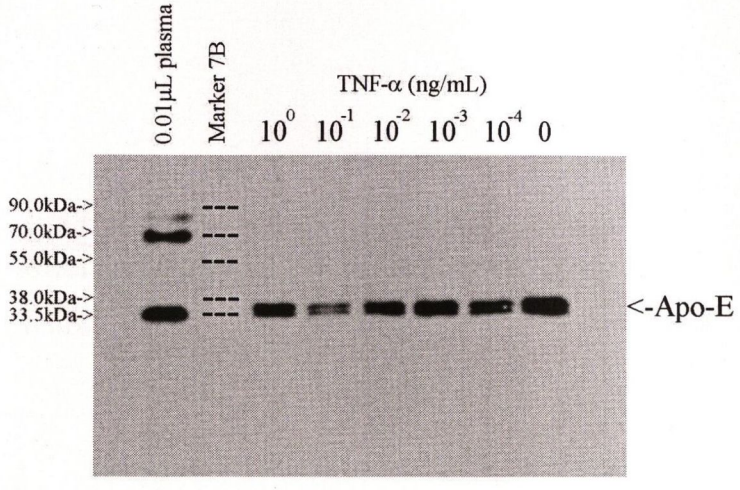

Figure 4.1.1b: Chemiluminescent staining of Western blot for Apo-E against cellular protein from TNF-α treated THP-1 cells: *Total cellular protein was extracted from a 5 day culture of THP-1 cells and 10µg loaded into a 15% PAGE SDS gel, electrophoresed, and transferred to nitrocellulose. This blot was then probed with anti-Apo-E antibody and visualised using a chemiluminescent substrate, as described in section 2.11.*

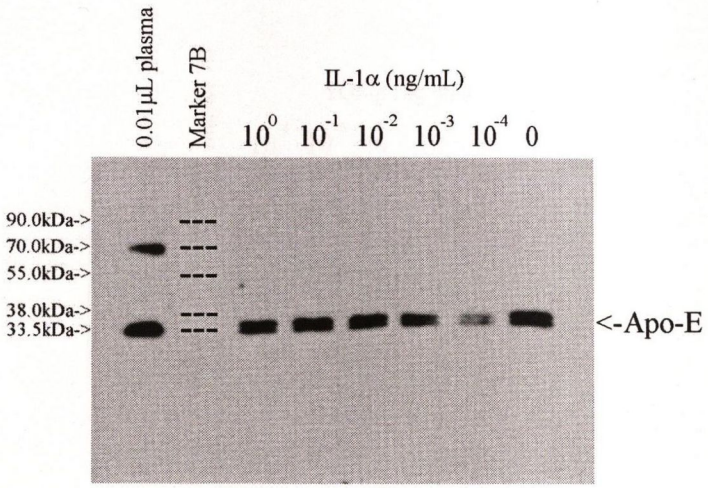

Figure 4.1.1c: Chemiluminescent staining of Western blot for Apo-E against cellular protein from IL-1α treated THP-1 cells: *Total cellular protein was extracted from a 5 day culture of THP-1 cells and 10µg loaded into a 15% PAGE SDS gel, electrophoresed, and transferred to nitrocellulose. This blot was then probed with anti-Apo-E antibody and visualised using a chemiluminescent substrate, as described in section 2.11.*

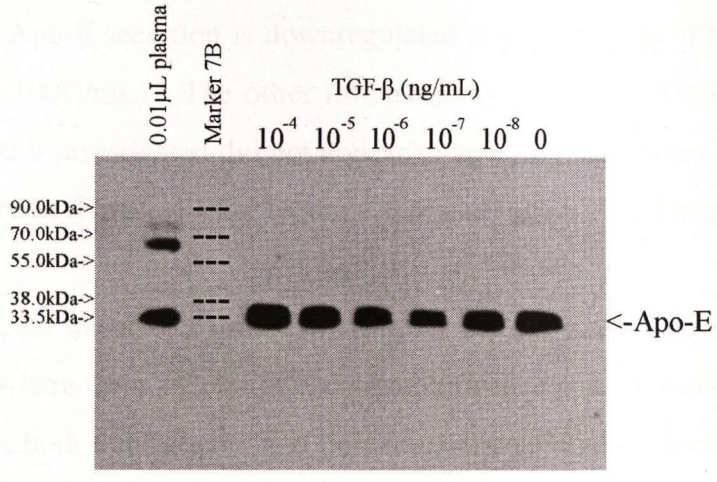

Figure 4.1.1d: Chemiluminescent staining of Western blot for Apo-E against cellular protein from TGF-β treated THP-1 cells: *Total cellular protein was extracted from a 5 day culture of THP-1 cells and 10µg loaded into a 15% PAGE SDS gel, electrophoresed, and transferred to nitrocellulose. This blot was then probed with anti-Apo-E antibody and visualised using a chemiluminescent substrate, as described in section 2.11.*

4.1.2 MODULATION OF APO-E SECRETION IN THP-1 CELLS BY CYTOKINES.

Only IFN-γ was shown to have any significant affect on Apo-E translation in THP-1 cells, but due to the various levels of control seen with Apo-E, it was thought necessary to examine the secretion of protein in each case. Concentrated medium was examined for Apo-E by SDS-PAGE followed by Western blotting. As can be seen in Figure 4.1.2a, Apo-E secretion is downregulated significantly by IFN-γ at 10ng/mL (equivalent to 100U/mL). The other inflammatory cytokines, TNF-α, IL-1α, and TGF-β, that were investigated did not appear to have any significant affect on levels of Apo-E secretion (Figure 4.1.2b, Figure 4.1.2c and Figure 4.1.2d respectively).

This would appear to conflict somewhat with the cellular extract experiments seen in section 4.1.1, where these cytokines had some influence at certain concentrations. It is possible that both translational and post-translational control mechanisms may be active in the case of these substances, as seen in some primary astrocyte experiments. In any case, the end physiological result, i.e. unaltered Apo-E secretion, would seem to render their action clinically irrelevant.

Table 4.1: The effects of inflammatory cytokines on THP-1 cellularand secreted Apo-E levels.

Cytokine	Effect on cellular Apo-E	Effect on secreted Apo-E
IFN-γ	inhibits	inhibits
TNF-α	none	none
IL-1α	none	none
TGF-β	none	none

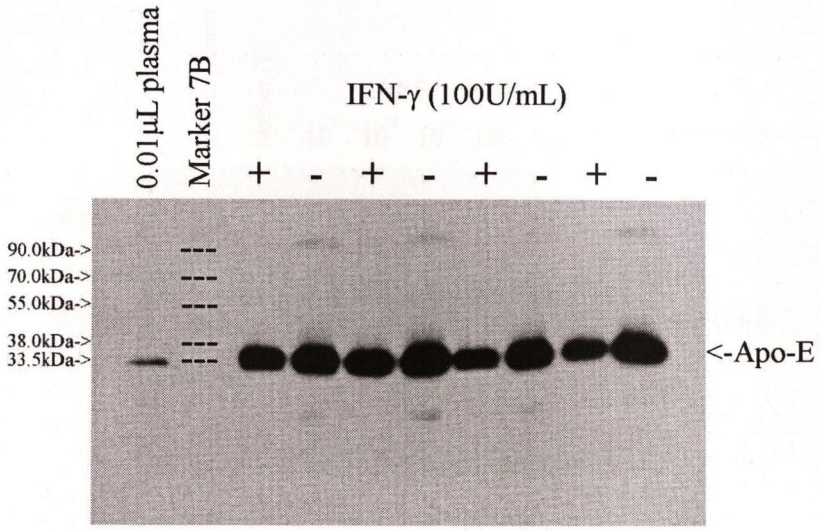

Figure 4.1.2a: Chemiluminescent staining of Western blot for Apo-E against secreted protein from IFN-γtreated THP-1 cells: *Concentrated secreted protein was prepared from a 5 day culture of THP-1 cells and 50µg loaded into a 15% PAGE SDS gel, electrophoresed, and transferred to nitrocellulose. This blot was then probed with anti-Apo-E antibody and visualised using a chemiluminescent substrate, as described in section 2.11.*

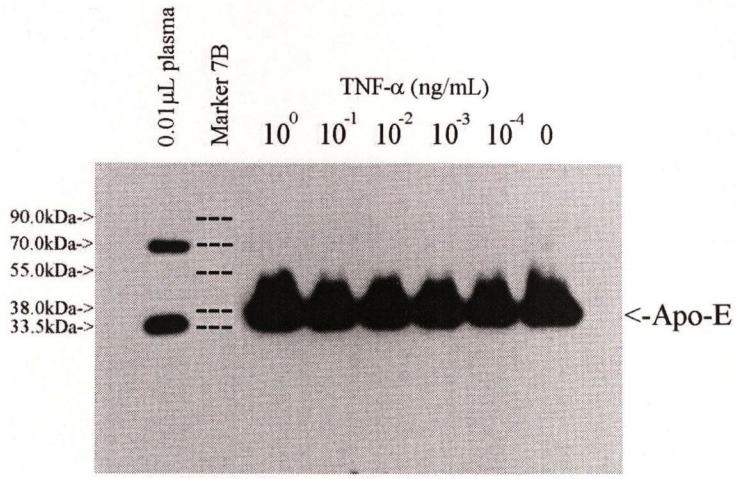

Figure 4.1.2b: Chemiluminescent staining of Western blot for Apo-E against secreted protein from TNF-α treated THP-1 cells: *Concentrated secreted protein was prepared from a 5 day culture of THP-1 cells and 50µg loaded into a 15% PAGE SDS gel, electrophoresed, and transferred to nitrocellulose. This blot was then probed with anti-Apo-E antibody and visualised using a chemiluminescent substrate, as described in section 2.11.*

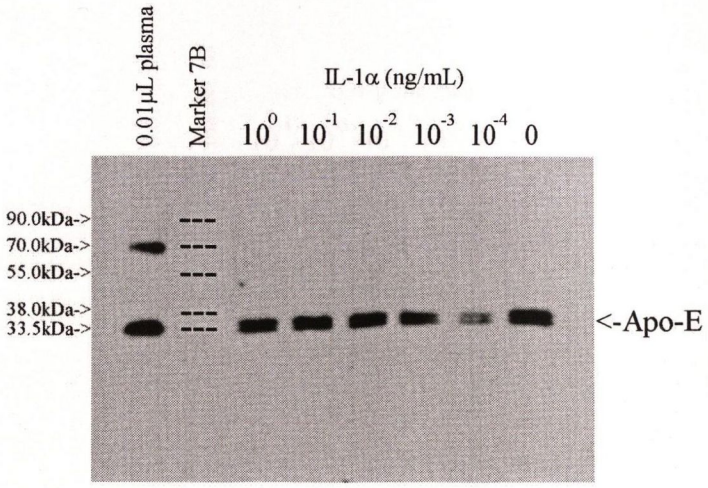

Figure 4.1.2c: Chemiluminescent staining of Western blot for Apo-E against secreted protein from IL-1α treated THP-1 cells: *Concentrated secreted protein was prepared from a 5 day culture of THP-1 cells and 50µg loaded into a 15% PAGE SDS gel, electrophoresed, and transferred to nitrocellulose. This blot was then probed with anti-Apo-E antibody and visualised using a chemiluminescent substrate, as described in section 2.11.*

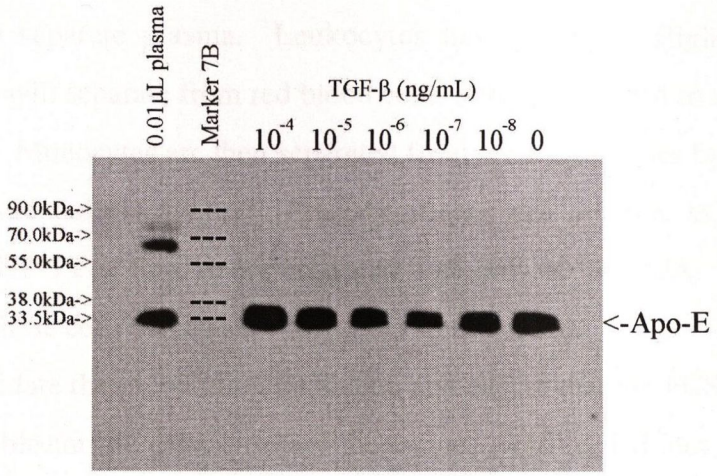

Figure 4.1.2d: Chemiluminescent staining of Western blot for Apo-E against secreted protein from TGF-β treated THP-1 cells: *Concentrated secreted protein was prepared from a 5 day culture of THP-1 cells and 50µg loaded into a 15% PAGE SDS gel, electrophoresed, and transferred to nitrocellulose. This blot was then probed with anti-Apo-E antibody and visualised using a chemiluminescent substrate, as described in section 2.11.*

4.2 DETECTION OF APO-E PROTEIN PRODUCTION IN PRIMARY MONOCYTE CELLS BY WESTERN BLOTTING.

Only IFN-γ was observed to have any affect on Apo-E protein levels in THP-1 cells, so it was deemed suitable to only examine the effects of this cytokine and IFN-β with respect to primary monocytes. Primary cell samples were received as 50mL buffy coats, which are the leukocyte enriched remainder after whole blood has been centrifuged to separate plasma. Leukocytes have a density slightly lower than 1.077g/mL, so will separate from red blood cells when centrifuged in a sugar solution at this density. Monocytes are then separated from other leukocytes by their ability to adhere to tissue culture plates. Primary monocytes are not as robust as the transformed THP-1 cell line, so are activated with only 10^{-8}M PMA. Once activated and adherent, these cells can be kept for weeks until needed.

In order to validate the preparation technique and ensure that the FCS replacer being used was suitable for the cells, primary monocytes were seeded into a 24-well plate and both cellular and concentrated medium samples were analysed for Apo-E protein using SDS-PAGE followed by Western blotting. The exposed film in Figure 4.2 shows that there is an accumulation of Apo-E protein in the medium over a course of 5 days

4.2.1 MODULATION OF APO-E TRANSLATION IN PRIMARY MONOCYTES BY CYTOKINES.

It was decided to set up a timecourse to look at the altered translation of Apo-E in cells under the influence of both IFN-β and IFN-γ. A 24-well plate was seeded with adherent monocytes, activated, and fed FCS free medium containing 100U/ml (10ng/mL) of IFN-γ. Total cellular protein samples were taken daily. As can be seen from Figure 4.2.1a, IFN-γ inhibits cellular levels of Apo-E for at least 6 days, with roughly the same amount of Apo-E protein present at each sampling. Suppression of Apo-E translation appears to be maximal approximately 3 days after treatment with IFN-γ.

When combinations of the two cytokines are used, as in Figure 4.2.1b, IFN-γ at 100U/mL appears to suppress Apo-E translation somewhat, whereas IFN-β at the same concentration appears to have little effect. Also, in the presence of both IFN-β and IFN-γ roughly the same downregulation of Apo-E translation is seen. Hence it can be surmised that IFN-β does not relieve IFN-γ induced repression of Apo-E translation.

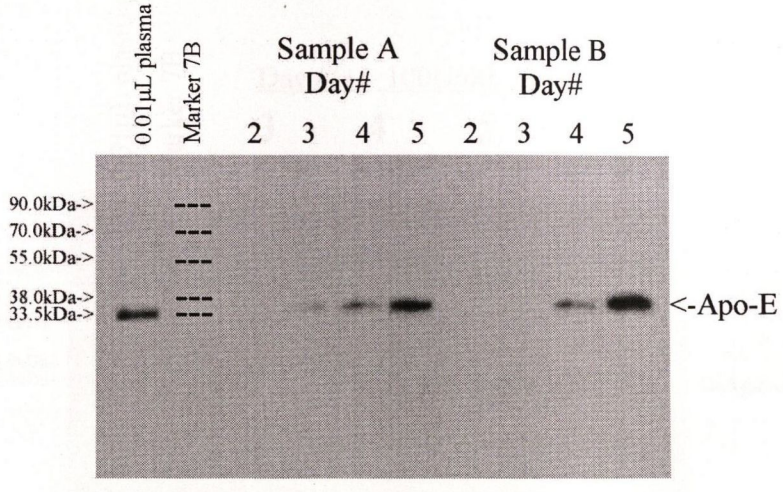

Figure 4.2: Chemiluminescent staining of Western blot for Apo-E against primary monocyte secreted protein: *Concentrated secreted protein was prepared from each day of a 5 day culture of primary monocyte cells and 50µg loaded into a 15% PAGE SDS gel, electrophoresed, and transferred to nitrocellulose. This blot was then probed with anti-Apo-E antibody and visualised using a chemiluminescent substrate, as described in section 2.11.*

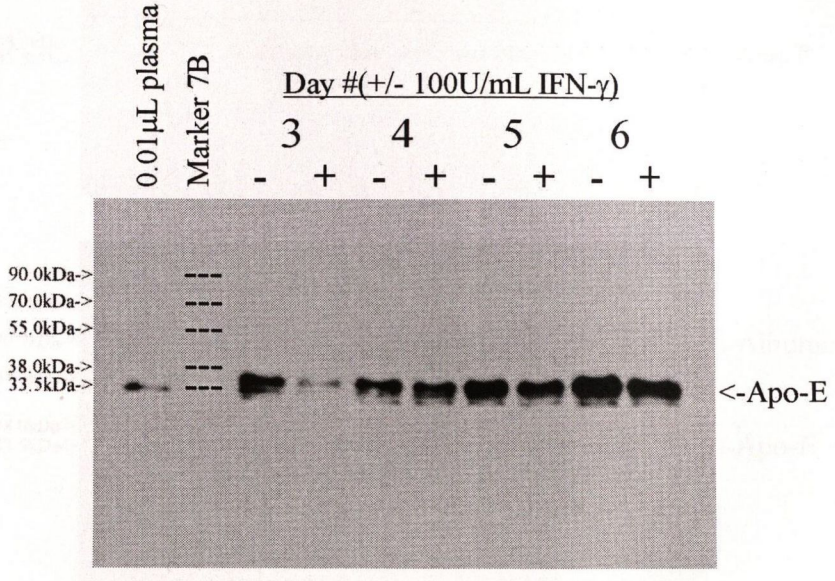

Figure 4.2.1a: Chemiluminescent staining of Western blot for Apo-E against cellular protein from IFN-γ treated primary monocyte cells: *Total cellular protein was extracted on each day of a 6 day culture of primary monocyte cells and 10µg loaded into a 15% PAGE SDS gel, electrophoresed, and transferred to nitrocellulose. This blot was then probed with anti-Apo-E antibody and visualised using a chemiluminescent substrate, as described in section 2.11.*

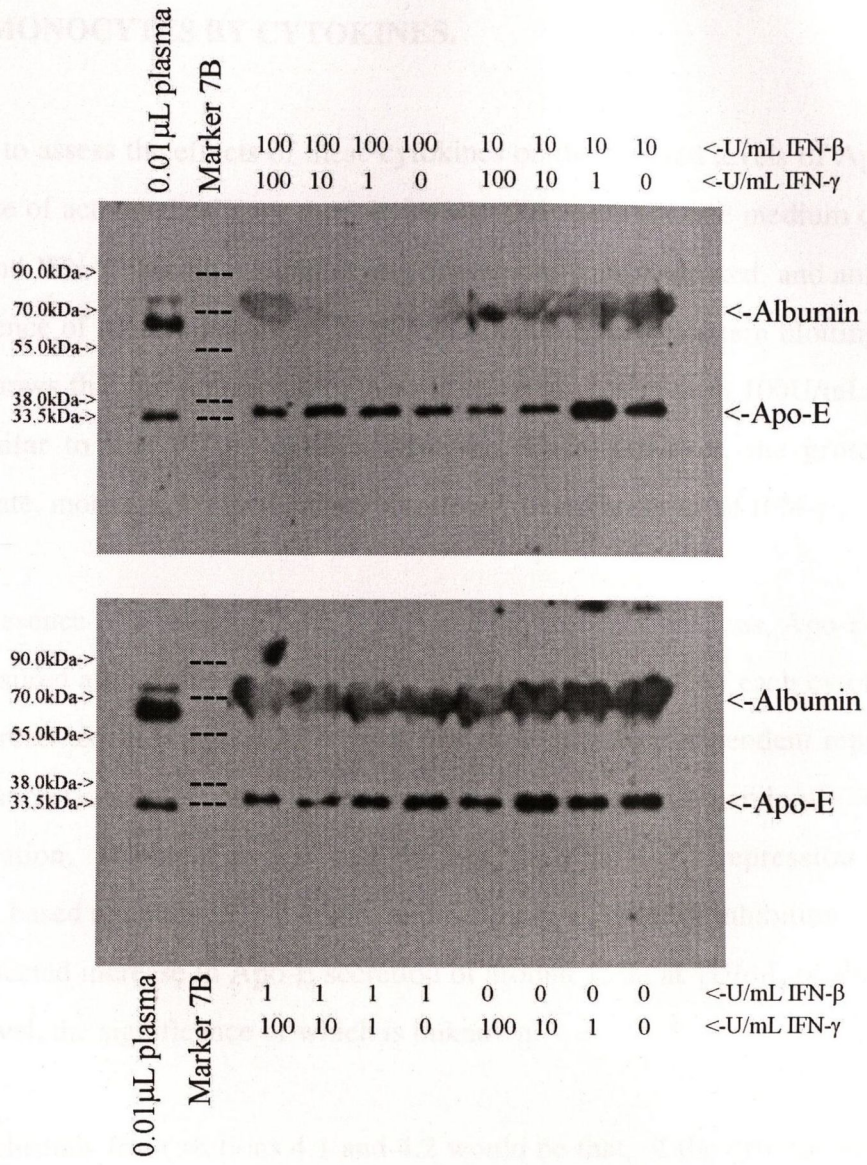

Figure 4.2.1b: Chemiluminescent staining of Western blot for Apo-E against cellular protein from IFN-γ and IFN-β treated primary monocyte cells: *Total cellular protein was extracted from a 5 day culture of primary monocyte cells and 10µg loaded into a 15% PAGE SDS gel, electrophoresed, and transferred to nitrocellulose. This blot was then probed with anti-Apo-E antibody and visualised using a chemiluminescent substrate, as described in section 2.11.*

4.2.2 MODULATION OF APO-E SECRETION IN PRIMARY MONOCYTES BY CYTOKINES.

In order to assess the effects of these cytokines on the secreted levels of Apo-E, a 24 well plate of activated primary monocytes was fed with FCS free medium containing IFN-β and IFN-γ. Medium samples were taken daily, concentrated, and analysed for the presence of Apo-E protein by SDS-PAGE followed by Western blotting. Figure 4.2.2a shows that the repression of Apo-E secreted protein with 100U/mL of IFN-γ was similar to that of the cellular protein. Here, however, the protein levels accumulate, more quickly in the controls, slower in the presence of IFN-γ.

In the presence of a range of both IFN-β and IFN-γ concentrations, Apo-E secretion was measured after a five day incubation with up to 100U/mL of each cytokine. The results presented in Figure 4.2.2b show that there is a dose dependent repression of Apo-E secretion by IFN-γ. This repression occurs independently of IFN-β concentration. It would appear that, in fact, there is also a repression of Apo-E secretion based upon the IFN-β levels, and certainly no relief of inhibition. There was an unexpected increase in Apo-E secretion of around 15% at 1U/mL of IFN-γ at any IFN-β level, the significance of which is unknown.

The conclusions from sections 4.1 and 4.2 would be that, of the cytokines examined, only IFN-γ appears to have any affect on Apo-E protein levels, and that IFN-β does not counteract this repression.

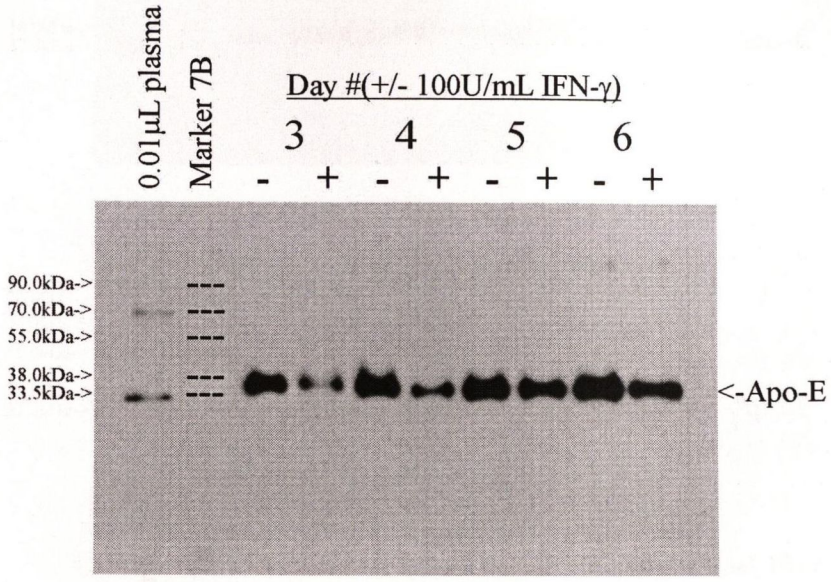

Figure 4.2.2a: Chemiluminescent staining of Western blot for Apo-E against secreted protein from IFN-γ treated primary monocyte cells: *Concentrated secreted protein was prepared from a 5 day culture of primary monocyte cells and 50µg loaded into a 15% PAGE SDS gel, electrophoresed, and transferred to nitrocellulose. This blot was then probed with anti-Apo-E antibody and visualised using a chemiluminescent substrate, as described in section 2.11.*

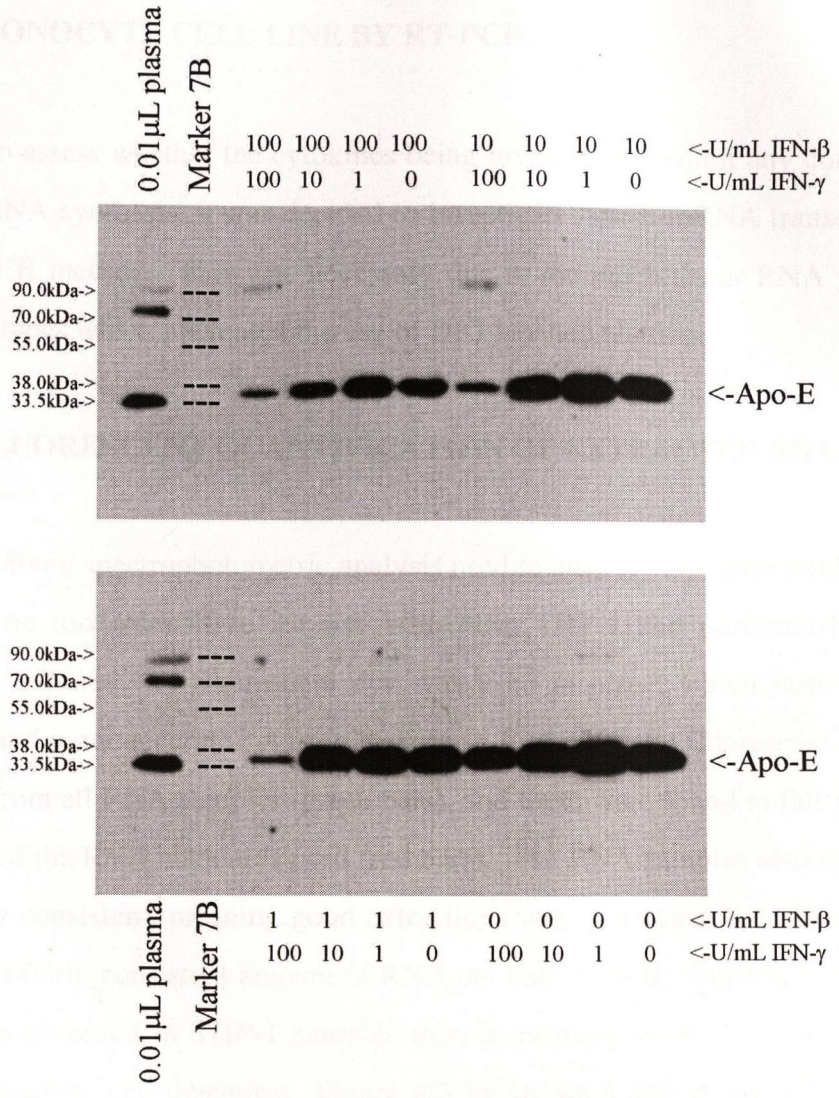

Figure 4.2.2b: Chemiluminescent staining of Western blot for Apo-E against secreted protein from IFN-γ and IFN-β treated primary monocyte cells: *Concentrated secreted protein was prepared from a 5 day culture of primary monocyte cells and 50µg loaded into a 15% PAGE SDS gel, electrophoresed, and transferred to nitrocellulose. This blot was then probed with anti-Apo-E antibody and visualised using a chemiluminescent substrate, as described in section 2.11.*

4.3 DETECTION OF APO-E mRNA PRODUCTION IN A THP-1 MONOCYTE CELL LINE BY RT-PCR.

In order to assess whether the cytokines being investigated exhibit any control at the level of RNA synthesis, it was decided to investigate Apo-E mRNA transcription by the RT-PCR method. This was necessary due to the much lower RNA yield from these cell types which prevented the use of DIG labelled blotting.

4.3.1 FLUORESCENT QUANTIFICATION OF EXTRACTED RNA.

The 260/280nm spectrophotometric analysis used to quantify astrocyte total RNA was found to be too insensitive for use with both THP-1 and particularly primary monocyte cultures. A fluorescent dye was used in place, which was both more sensitive and more accurate. As can be seen in Figure 4.3.1a, fluorescent signal was obtained from all RNA samples (green bars), and these were found to fall well within the range of the RNA standards used (red bars). The RNA samples also appear to be reasonably consistent, meaning good extractions were performed and that there was generally a fairly consistent amount of RNA per culture well. Generally, much more signal was observed in THP-1 samples than in primary monocyte cultures due to differing starting cell densities. Figure 4.3.1b shows a plot of the RNA standard concentration against the fluorescence measured for each dilution. This shows that there is a very linear relationship between RNA quantity and fluorescence using the RIBO-Green assay.

After quantification, all RNA samples were adjusted to 100pg/μL, and triplicate samples pooled.

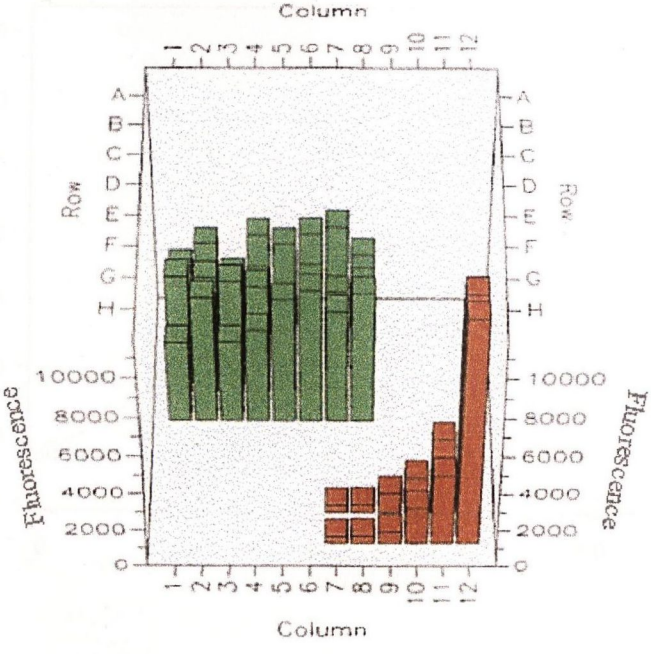

Figure 4.3.1a: Fluorescent plate readings from RIBO-Green assay of primary monocyte and standard RNA: *RNA standard stock (red) and RNA isolated from primary monocyte cells (green) were mixed with RIBO-Green dye and the plate read in a fluorescent plate reader set to excite at 500nm and read at 525nm, as detailed in section 2.7.4. The readings obtained were then plotted graphically.*

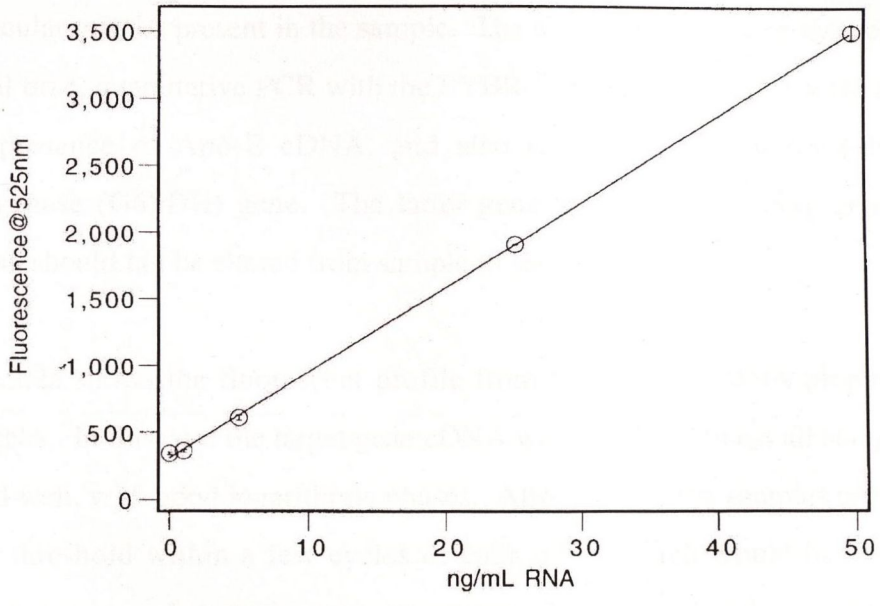

Figure 4.3.1b: RNA fluorescence standard curve: *1mg/mL RNA standard stock was diluted to give a range of 0ng/mL to 50ng/mL and assayed using the RIBO-Green fluorescent dye. Readings were graphed as fluorescence at 525nm versus concentration of RNA, as detailed in section 2.7.4.*

4.3.2 MODULATION OF APO-E TRANSCRIPTION IN THP-1 CELLS BY CYTOKINES.

Using the same quantity of total RNA, cDNA was prepared from each pooled sample using the reverse transcriptase reaction described in section 2.12.1. Since all these reactions were allowed to proceed for exactly 60 minutes, and all with the same starting RNA, the cDNA generated should be representative of the original amount of any particular mRNA present in the sample. The cDNA samples were then examined using real time, quantitative PCR with the SYBR-Green dye. Samples were all tested for the presence of Apo-E cDNA, and also tested for the glucose-6-phophate dehydrogenase (G6PDH) gene. The latter gene was a housekeeping gene whose expression should not be altered from sample to sample.

Figure 4.3.2a shows the fluorescent profile from the PCR of cDNA prepared from THP-1 RNA. In this case the target gene cDNA was Apo-E. Almost all samples have amplified well, with good logarithmic phases. Also, most of the samples cross the set intensity threshold within a few cycles of each other, which would be expected if there were no gross changes in gene expression. To ensure that the amplification is running smoothly, a dissociation curve was performed after the PCR has been completed. Here the inverse of signal intensity is compared to temperature. As can be seen in Figure 4.3.2b, almost all the samples consist only of single stranded RNA at around 88^{o}C. Any samples that did not dissociate at this temperature were discarded and those results ignored.

Similarly, using primers for the G6PDH gene, Figure 4.3.2c shows that almost all samples have good logarithmic phases of amplification. Also, here we can see that the fluorescent intensity of almost every sample crosses the set threshold at almost exactly the same point, within 1 amplification cycle of each other. The dissociation curve observed for all of these samples were also good (Figure 4.3.2d), showing a maximum amount of denatured, single stranded DNA, at around 88^{o}C.

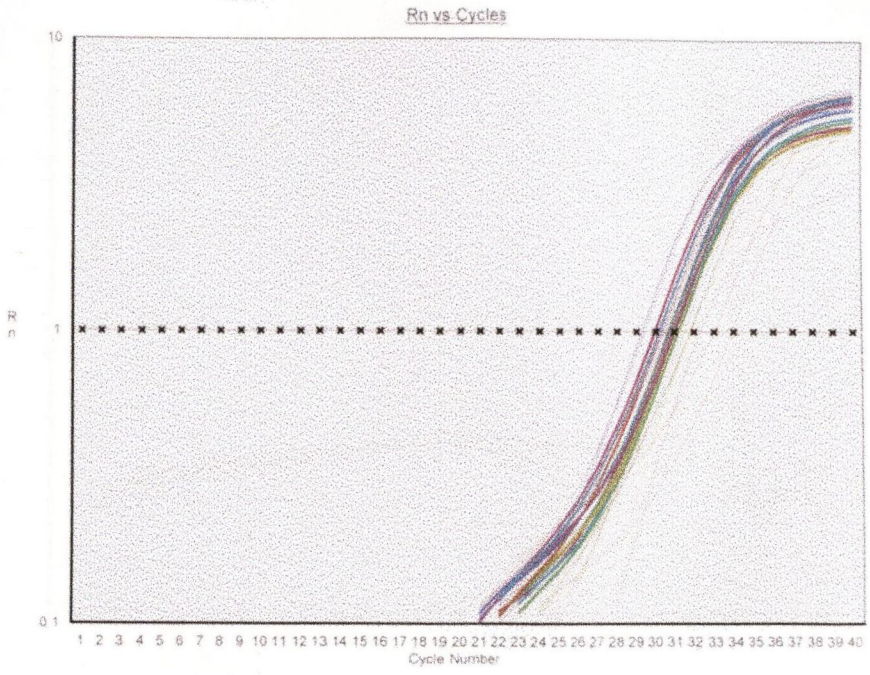

Figure 4.3.2a: Plot of SYBR-Green fluorescence against PCR cycle with Apo-E gene as target: *cDNA, corresponding to 250pg of THP-1 mRNA treated with cytokines, was amplified in the presence of SYBR-Green dye using primers specific for the Apo-E gene. The fluorescence measured at each amplification was then plotted against the cycle number, as described in section 2.12.*

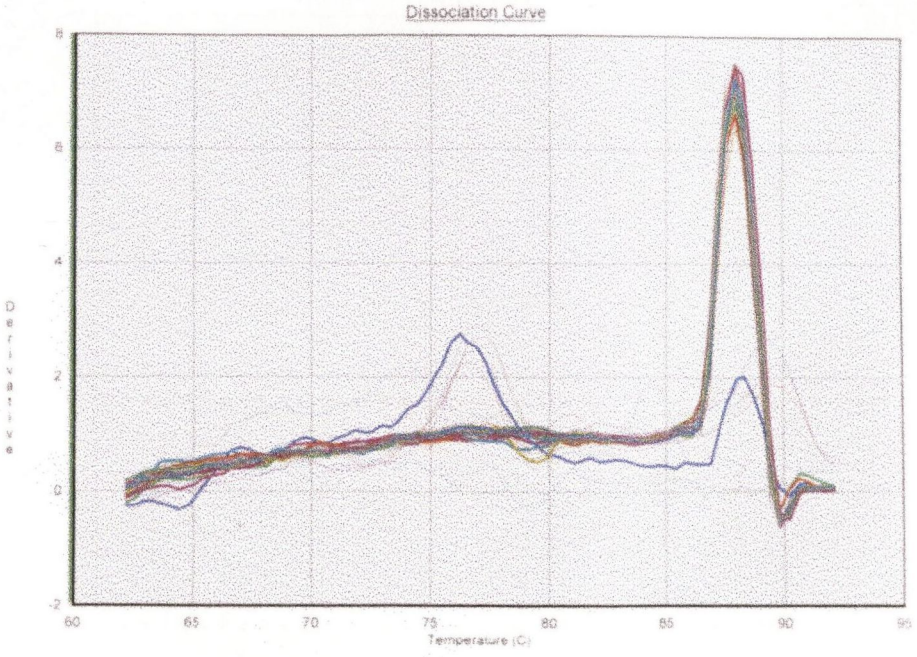

Figure 4.3.2b: Plot of SYBR-Green fluorescence derivative against temperature with Apo-E gene as target: *After amplification of Apo-E cDNA, samples were heated incrementally from 60°C to 95°C. The derivative of the fluorescence measured at each increment was then plotted against the temperature, as described in section 2.12.*

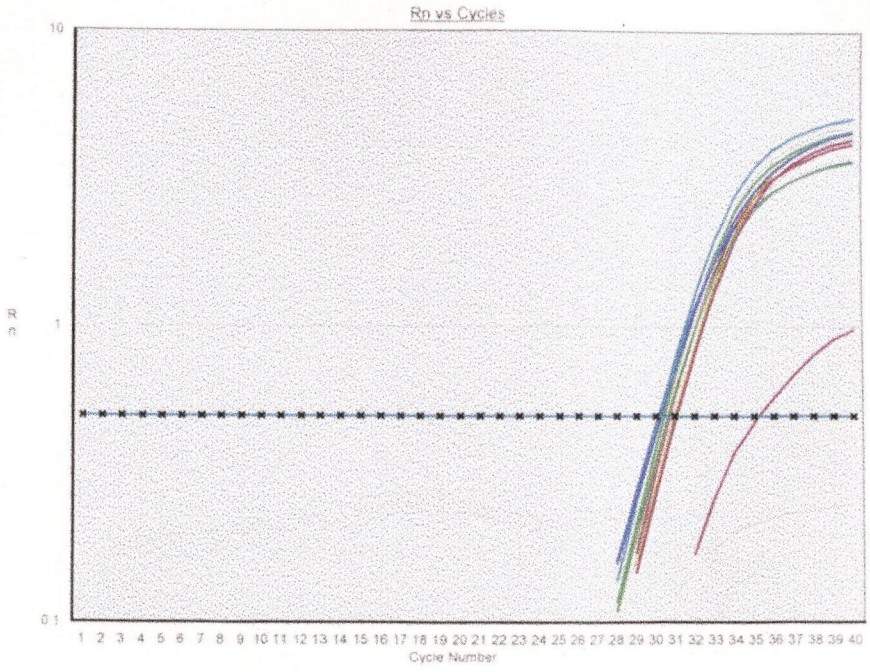

Figure 4.3.2c: Plot of SYBR-Green fluorescence against PCR cycle with G6PDH gene as target: *cDNA, corresponding to 250pg of THP-1mRNA treated with cytokines, was amplified in the presence of SYBR-Green dye using primers specific for the G6PDH gene. The fluorescence measured at each amplification was then plotted against the cycle number, as described in section 2.12.*

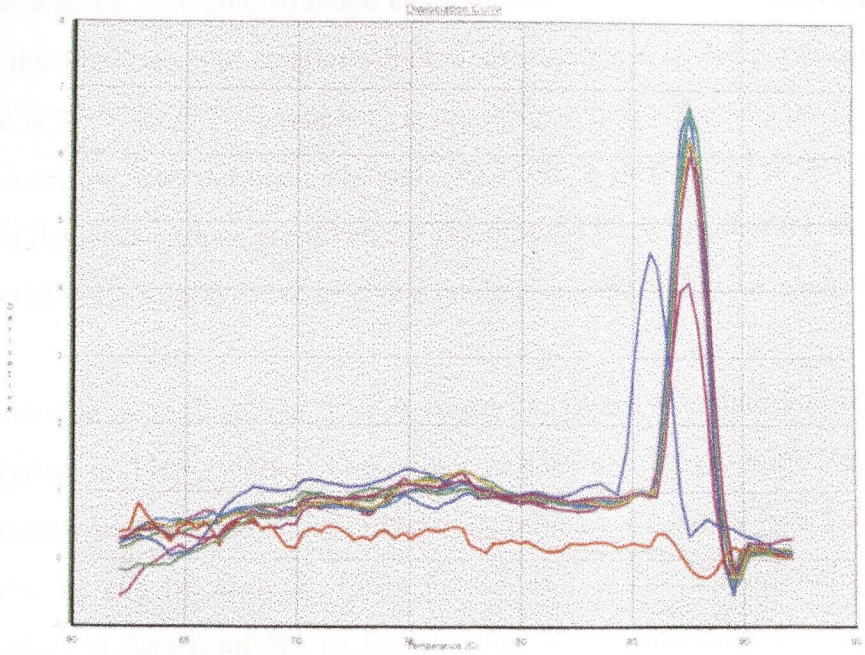

Figure 4.3.2d: Plot of SYBR-Green fluorescence derivative against temperature with G6PDH gene as target: *After amplification of G6PDH cDNA, samples were heated incrementally from 60°C to 95°C. The derivative of the fluorescence measured at each increment was then plotted against the temperature, as described in section 2.12.*

THP-1 cells were examined for Apo-E mRNA expression under the influence of a number of cytokines. THP-1 suspension cultures were activated and allowed to adhere in 24 well plates at 1mL per plate, and treated with a range of concentrations of each cytokine. After 5 days total RNA was isolated and examined for Apo-E mRNA using real time RT-PCR.

Since the amount of double-stranded DNA present at each cycle of the PCR is double that of the previous, the relative amount of starting Apo-E template to G6PDG template is $2^{Ct(G6PDH)}/2^{Ct(Apo\text{-}E)}$, where C_t is the cycle at which that sample's fluorescence crosses a certain, user defined threshold. This can be abbreviated to $2^{Ct(G6PDH) - Ct(Apo\text{-}E)}$. Standard deviation values are given for the original C_t values, not for the calculated exponentials, and so only serve to give a guide to reproducibility between PCR runs.

As can be seen in Table 4.3.3a, TNF-α appears to have a stimulatory effect on Apo-E transcription in THP-1 cells. This effect is dose dependent, with almost 10 fold production at 1ng/mL, and is not linked to increased cytotoxicity (see Table 4.5.1(I)). Similarly, as can be seen in Table 4.3.3b, IL-1α has a stimulatory effect on transcription of Apo-E mRNA in THP-1 cells. This is also dose dependent, and not linked to increased LDH levels in the medium (see Table 4.5.1(I)). TGF-β, another pro-inflammatory cytokine, also appears to significantly increase Apo-E transcription, as can be seen in Table 4.3.3c. Again there is dose dependency, but at the TGF-β concentrations examined there was a large increase in LDH activity (see Table 4.5.1(I)), leading one to believe that this is a phenomenon associated with cytotoxicity. These results are all unusual in that there appeared to be no change in Apo-E protein translation or secretion from THP-1 cells, as demonstrated by the results seen earlier in Section 4.1.

In the presence of both IFN-β and IFN-γ, the results shown in Table 4.3.3d would indicate that both cytokines have an inhibitory effect on Apo-E mRNA production in THP-1 cells. IFN-β does not appear to counteract IFN-γ induced Apo-E mRNA

inhibition, and in the presence of both cytokines there is an even greater reduction in transcription.

Table 4.3.3a: The modulation of Apo-E mRNA expression in THP-1 cells by TNF-α.

TNF-α (ng/mL)	1	0.1	0.01	0.001	0.0001	0
C_t Apo-E	25.28	24.96	24.98	25.22	25.26	26.15
SD	0.299	0.146	0.293	0.238	0.366	0.311
C_t G6PDH	30.58	29.18	28.94	28.35	27.60	27.98
SD	0.313	0.126	0.382	0.255	0.255	0.245
2^{G-A}	39.40	18.64	15.56	8.75	5.06	3.55
%Change	+910%	+425%	+338%	+146%	+43%	100%

RNA pooled from triplicate 5 day, TNF-α treated, THP-1 cultures was converted to cDNA, and examined for Apo-E cDNA using the SYBR-Green real time PCR system. The cycle at which fluorescence crossed a set point was calculated, and compared with that of the housekeeping gene G6PDH, as described in section 2.12.

Table 4.3.3b: The modulation of Apo-E mRNA expression in THP-1 cells by IL-1α.

IL-1α (ng/mL)	1	0.1	0.01	0.001	0.0001	0
C_t Apo-E	25.79	26.57	26.04	25.10	26.09	26.14
SD	0.255	0.182	0.315	0.232	0.311	0.323
C_t G6PDH	29.98	31.05	28.62	27.88	27.69	27.60
SD	0.135	0.302	0.353	0.161	0.390	0.325
2^{G-A}	18.25	22.32	5.98	6.87	3.03	2.75
%Change	+564%	+712%	+117%	+150%	+10%	100%

RNA pooled from triplicate 5 day, IL-1α treated, THP-1 cultures was converted to cDNA, and examined for Apo-E cDNA using the SYBR-Green real time PCR system. The cycle at which fluorescence crossed a set point was calculated, and compared with that of the housekeeping gene G6PDH, as described in section 2.12.

Table 4.3.3c: The modulation of Apo-E mRNA expression in THP-1 cells by TGF-β.

TGF-β (ng/mL)	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	0
C_t Apo-E	32.13	31.36	30.92	31.32	29.51	30.53
SD	0.133	0.263	0.219	0.163	0.215	0.290
C_t G6PDH	35.23	34.17	33.47	33.37	31.07	31.05
SD	0.158	0.384	0.334	0.392	0.338	0.369
2^{G-A}	8.55	7.02	5.87	4.13	2.95	1.43
%Change	+498%	+391%	+310%	+189%	+106%	100%

RNA pooled from triplicate 5 day, TGF-β treated THP-1 cultures was converted to cDNA, and examined for Apo-E cDNA using the SYBR-Green real time PCR system. The cycle at which fluorescence crossed a set point was calculated, and compared with that of the housekeeping gene G6PDH, as described in section 2.12.

Table 4.3.3d(I): The modulation of Apo-E mRNA expression in THP-1 cells by IFN-β and IFN-γ (calculated ratios).

%Change in Apo-E mRNA.

IFN-γ(U/mL)→ IFN-β(U/mL)↓	100	10	0
100	-31%	-30%	-34%
10	-30%	-40%	+2%
0	-12%	-22%	100%

2^{G-A}.

IFN-γ(U/mL)→ IFN-β(U/mL)↓	100	10	0
100	1.51	1.53	1.45
10	1.47	1.31	2.25
0	1.94	1.72	2.20

RNA pooled from triplicate 5 day, IFN-β and IFN-γ treated THP-1 cultures was converted to cDNA, and examined for Apo-E cDNA using the SYBR-Green real time PCR system. The cycle at which fluorescence crossed a set point was calculated, and compared with that of the housekeeping gene G6PDH, as described in section 2.12.

Table 4.3.3d(II): The modulation of Apo-E mRNA expression in THP-1 cells by IFN-β and IFN-γ (C_t values).

Mean C_t for Apo-E target.

IFN-γ(U/mL)→ IFN-β(U/mL)↓	100	10	0
100	30.99	30.71	30.76
10	31.54	30.82	30.38
0	30.86	30.55	30.59

Mean C_t for G6PDH target.

IFN-γ(U/mL)→ IFN-β(U/mL)↓	100	10	0
100	31.58	31.32	31.30
10	32.10	31.21	31.55
0	31.82	31.33	31.73

RNA pooled from triplicate 5 day, IFN-β and IFN-γ treated THP-1 cultures was converted to cDNA, and examined for Apo-E cDNA using the SYBR-Green real time PCR system. The cycle at which fluorescence crossed a set point was calculated, and compared with that of the housekeeping gene G6PDH, as described in section 2.12.

Table 4.3.3d(III): The modulation of Apo-E mRNA expression in THP-1 cells by IFN-β and IFN-γ (standard deviations).

SD of C_t for Apo-E target.

IFN-γ(U/mL)→ IFN-β(U/mL)↓	100	10	0
100	0.360	0.181	0.355
10	0.100	0.300	0.362
0	0.317	0.131	0.400

SD of C_t for G6PDH target.

IFN-γ(U/mL)→ IFN-β(U/mL)↓	100	10	0
100	0.298	0.272	0.270
10	0.224	0.380	0.294
0	0.371	0.381	0.143

RNA pooled from triplicate 5 day, IFN-β and IFN-γ treated THP-1 cultures was converted to cDNA, and examined for Apo-E cDNA using the SYBR-Green real time PCR system. The cycle at which fluorescence crossed a set point was calculated, and compared with that of the housekeeping gene G6PDH, as described in section 2.12.

4.4 DETECTION OF APO-E mRNA PRODUCTION IN PRIMARY MONOCYTE CELLS BY RT-PCR.

To investigate the transcription of Apo-E mRNA in primary cells, leukocyte enriched buffy coats were used to prepare monolayers of activated monocytes. These cells were treated with cytokines, and RNA examined for Apo-E transcription by RT-PCR, by identical methods to those used for THP-1 cells.

4.4.1 MODULATION OF APO-E TRANSCRIPTION IN PRIMARY MONOCYTES BY CYTOKINES.

It was decided to examine primary monocytes for influence only by IFN-β and IFN-γ, as these were thought to be the cytokines most central to the project theory. Due to the likelihood of primary cells being much more sensitive to these substances, an extra ten-fold dilution of the cytokines was also examined.

Th results shown in Table 4.4.1 indicate that both IFN-β and IFN-γ have profound effects on Apo-E transcription. Both appear to exhibit dose dependent repression of Apo-E transcription, with a drop of 85% seen with 100U/mL of IFN-β and an 80% drop with 100U/ml of IFN-γ. This repression was associated with only a slight increase in LDH activity in the case of IFN-β (see section 4.5.2). In combination, there is an even greater reduction in transcription, down by 93% in the presence of 100U/mL of both cytokines. The conclusions from these results would be that not only does IFN-β fail to relieve IFN-γ induced Apo-E downregulation, it also increases this suppression in a dose dependent manner.

Table 4.4.1(I): The modulation of Apo-E mRNA expression in primary monocyte cells by IFN-β and IFN-γ (calculated ratios).

%Change in Apo-E mRNA.

IFNγ(U/mL)→ IFN-β(U/mL)↓	100	10	1	0
100	-93%	-86%	-89%	-85%
10	-92%	-60%	-59%	-42%
1	-55%	-31%	-59%	-4%
0	-80%	+21%	-19%	100%

2^{G-A}.

IFNγ(U/mL)→ IFN-β(U/mL)↓	100	10	1	0
100	0.120	0.239	0.189	0.261
10	0.140	0.678	0.688	0.979
1	0.758	1.173	0.688	1.624
0	0.339	2.042	1.366	1.693

RNA pooled from triplicate 5 day, IFN-β and IFN-γ treated primary monocyte cultures was converted to cDNA, and examined for Apo-E cDNA using the SYBR-Green real time PCR system. The cycle at which fluorescence crossed a set point was calculated, and compared with that of the housekeeping gene G6PDH, as described in section 2.12.

Table 4.4.1(II): The modulation of Apo-E mRNA expression in primary monocyte cells by IFN-β and IFN-γ (C_t values).

Mean C_t for Apo-E target.

IFNγ(U/mL)→ IFN-β(U/mL)↓	100	10	1	0
100	31.31	30.65	30.75	30.60
10	31.35	28.96	29.26	28.25
1	29.90	28.15	28.93	27.62
0	30.46	27.36	27.85	27.51

Mean C_t for G6PDH target.

IFNγ(U/mL)→ IFN-β(U/mL)↓	100	10	1	0
100	28.25	28.59	28.35	28.66
10	28.51	28.40	28.72	28.22
1	29.50	28.38	28.39	28.32
0	28.90	28.39	28.30	28.27

RNA pooled from triplicate 5 day, IFN-β and IFN-γ treated primary monocyte cultures was converted to cDNA, and examined for Apo-E cDNA using the SYBR-Green real time PCR system. The cycle at which fluorescence crossed a set point was calculated, and compared with that of the housekeeping gene G6PDH, as described in section 2.12.

Table 4.4.1(III): The modulation of Apo-E mRNA expression in primary monocyte cells by IFN-β and IFN-γ (standard deviations).

SD of C_t for Apo-E target.

IFNγ(U/mL)→ IFN-β(U/mL)↓	100	10	1	0
100	0.444	0.219	0.432	0.362
10	0.455	0.355	0.155	0.451
1	0.349	0.165	0.355	0.264
0	0.363	0.275	0.120	0.359

SD of C_t for G6PDH target.

IFNγ(U/mL)→ IFN-β(U/mL)↓	100	10	1	0
100	0.347	0.129	0.279	0.354
10	0.165	0.399	0.337	0.118
1	0.387	0.235	0.203	0.365
0	0.151	0.207	0.270	0.126

RNA pooled from triplicate 5 day, IFN-β and IFN-γ treated primary monocyte cultures was converted to cDNA, and examined for Apo-E cDNA using the SYBR-Green real time PCR system. The cycle at which fluorescence crossed a set point was calculated, and compared with that of the housekeeping gene G6PDH, as described in section 2.12.

4.5 LDH CYTOTOXICITY ASSAY ON THP-1 AND PRIMARY MONOCYTE EXPERIMENTS.

As with the astrocytoma experiments, it was necessary to assess whether cytotoxicity was playing a role in the regulation of Apo-E protein signal detected from both the THP-1 and primary monocyte cells investigated. After activation with PMA, both of these cell types tend to be quite long lived, as they begin to granulate and adhere with collagen in place of the fibronectin normally found in matrix attachment proteins. The DMSO used to dilute PMA is also very toxic to THP-1 cell lines, so it was important to note any excessive LDH levels in activated cells. 50μL of each medium sample was thawed and centrifuged for 5 minutes in a Beckman microfuge to remove cellular debris, and examined for LDH activity as described in section 2.14.

When an LDH standard was examined, 50μl of LDH at 3.3U/mL (= 0.165U) corresponded to an absorbance of 0.146 at 490nm.

4.5.1 CYTOTOXICITY IN THP-1 MONOCYTE CULTURES.

The results shown in Table 4.5.1(I) show that neither TNF-α nor IL-1α exhibited any adverse effect on medium LDH levels, and were therefore assumed to be non-cytotoxic to the THP-1 cell line at the cytokine concentrations used. TGF-β was shown to have dose-dependent cytotoxic effects on the cells, with an almost doubling of LDH levels found in the medium. The results shown in Table 4.5.1(II) indicate that IFN-β is non-cytotoxic to THP-1 cells at up to 100U/mL, whereas there was a 41% increase in LDH activity at the same levels of IFN-γ. It can be concluded that some of the Apo-E mRNA repression and stimulation, seen with TGF-β and IFN-γ respectively, may be due to their cytotoxic effects on the THP-1 cell line.

Table 4.5.1(I): LDH cytotoxicity assay on THP-1 experiments.

TNF-α (ng/mL)	1	0.1	0.01	0.001	0.0001	0
Mean Abs @490nm	0.999	1.021	0.959	0.994	1.086	1.048
SD	0.025	0.028	0.027	0.093	0.091	0.053

IL-1α (ng/mL)	1	0.1	0.01	0.001	0.0001	0
Mean Abs @490nm	1.191	0.915	1.010	1.103	1.074	1.200
SD	0.124	0.151	0.055	0.053	0.009	0.084

TGF-β (ng/mL)	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	0
Mean Abs @490nm	1.196	1.089	0.831	0.589	0.554	0.583
SD	0.089	0.079	0.029	0.077	0.070	0.083

50μL (or 20μl for TGF-β) of thawed, cell free, medium was used in conjunction with the Cytotox LDH assay kit, and absorbancies read at 490nm. Higher readings indicate more cytotoxicity as LDH is released from cells. All samples are taken in triplicate, as described in section 2.14.

Table 4.5.1(II): LDH cytotoxicity assay on THP-1 experiments.

IFN-β (U/mL)	100	10	0
Mean Abs@490nm	1.079	1.098	1.055
SD	0.104	0.037	0.152

IFN-γ (U/mL)	100	10	0
Mean Abs@490nm	1.426	0.992	1.012
SD	0.112	0.023	0.133

50μL of thawed, cell free, medium was used in conjunction with the Cytotox LDH assay kit, and absorbancies read at 490nm. Higher readings indicate more cytotoxicity as LDH is released from cells. All samples are taken in triplicate, as described in section 2.14.

4.5.2 CYTOTOXICITY IN PRIMARY MONOCYTE CULTURES.

The results shown in Table 4.5.2 would indicate a much lower general amount of LDH activity in primary monocyte experiments than in the THP-1 cell line. This was probably due to the much lower amount of cells per well at the starting point of a primary culture experiment. Neither IFN-β nor IFN-γ were seen to be significantly cytotoxic to PBMC cells at the concentrations used although a slight drop in LDH activity was noted with 10U/mL of IFN-γ, the same concentration at which a 21% increase in Apo-E mRNA was noted in primary monocytes (see Table 4.5.1(II)).

Table 4.5.2: LDH cytotoxicity assay on primary monocyte experiments:

IFN-β (U/mL)	100	10	1	0
Mean Abs@490nm	0.522	0.477	0.491	0.454
SD	0.049	0.085	0.077	0.082

IFN-γ (U/mL)	100	10	1	0
Mean Abs@490nm	0.479	0.371	0.464	0.412
SD	0.082	0.057	0.062	0.070

20μL of thawed, cell free, medium was used in conjunction with the Cytotox LDH assay kit, and absorbancies read at 490nm. Higher readings indicate more cytotoxicity as LDH is released from cells. All samples are taken in triplicate, as described in section 2.14.

CHAPTER 5

APO-E GENOTYPE AND MONOCYTE PRODUCTION OF APO-E PROTEIN.

After ascertaining the effects of several inflammatory cytokines and the drug IFN-β on Apo-E production by PBMCs, it was decided to investigate the link between Apo-E genotype and the modulations seen. Some (although not all) reports have suggested a link between rate of disability progression and Apo-E genotype [Chapman et al 1999, Evangelou et al 1999, Olivieri et al 1999 and Gaillard et al 1998], with the ε4 allele implicated as deleterious to disease outcome. The aims of the experiments presented here were to assess whether Apo-E modulation in macrophages by IFN-γ and IFN-β are a function of the Apo-E genotype, and thus gain some understanding of the effects that may be being observed epidemiologically in MS patients. The amounts of Apo-E present in donor plasma were also quantified in order to show reproducibility between Western blots where different standards may have been used (repeated freeze thaw cycles may have degraded Apo-E in the donor plasma). Some information may also be gathered concerning Apo-E levels in normal plasma, although this is obviously not all from a monocyte/macrophage source.

The results obtained were presented under the following headings:

5.1 PCR of Apo-E gene in donor buffy coats.

5.2 Distribution of ε2, ε3 and ε4 alleles in donor buffy coats.

5.3 The relationship between Apo-E genotype and Apo-E levels in plasma and primary monocyte cell culture medium.

5.1 PCR OF APO-E GENE IN DONOR BUFFY COATS.

On receiving donor buffer coats, 50μl was set aside and frozen for use in genotyping. The system used here for genotyping was that of Scanu et al 1991, in this case altered slightly to accommodate the leukocyte, and therefore DNA rich, buffy coats.

5.1.1 DETECTION OF APO-E AMPLICON.

To optimise the PCR process, a range of $MgCl_2$ was used as a co-factor in the PCR mix. $MgCl_2$ is a vital cofactor for the DNA polymerase enzyme, and may be required in higher concentrations where there is more starting DNA. Thawed buffy coats were diluted 1 in 10 with distilled water, and 0.5µL used as template in a final reaction volume of 25µL, and products examined using PAGE-TBE electrophoresis. Figure 5.1.1 shows that strong, 227bp PCR product bands are obtained from buffy coats, with the greatest amount of product being seen at an $MgCl_2$ of 25mM. This concentration of $MgCl_2$ was used for all further amplifications. Also there are no extra bands present, indicating good specificity for the Apo-E gene at the annealing temperature used.

5.1.2 DETECTION OF DIGESTED AMPLICON FRAGMENTS.

All the buffy coats received for use in primary culture were amplified for the Apo-E target, and subsequently digested with the Cfo I restriction endonuclease to give the fragments indicative of each genotype. These fragments were then examined using PAGE-TBE electrophoresis. The gel pictured in Figure 5.1.2 shows that all PCR products have digested, as there is no 227bp product remaining in any lane. All samples also have the required fragments needed for correct genotyping, with no extra bands visible.

5.1.3 PCR OF GENOTYPE REFERENCE STANDARDS.

In order to validate the PCR method used, DNA extracted from the blood of donors of known genotype was used as template for amplification, and subsequently digested with Cfo I. The fragments were then examined using PAGE-TBE electrophoresis. Figure 5.1.3 shows that all 6 genotypes are clearly distinguishable, with no extra bands in any lanes, and with a pattern identical to the fragment schematic shown in Figure 2.5.

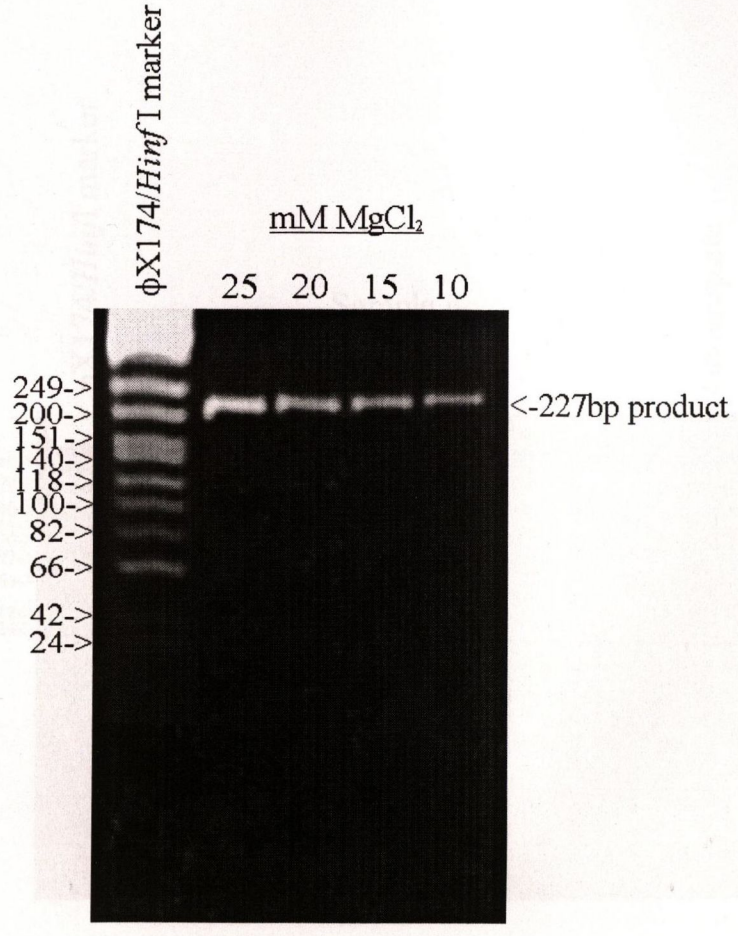

Figure 5.1.1: Buffy coat DNA amplified for the Apo-E gene electrophoresed in 20% PAGE-TBE gel: *0.5µl of 1 in 10 diluted donor buffy coat was amplified using primers for a 227bp section of the Apo-E gene using a range of $MgCl_2$ concentrations. The product was then electrophoresed in a 20% PAGE-TBE gel, and visualised under UV light, as described in section 2.13.*

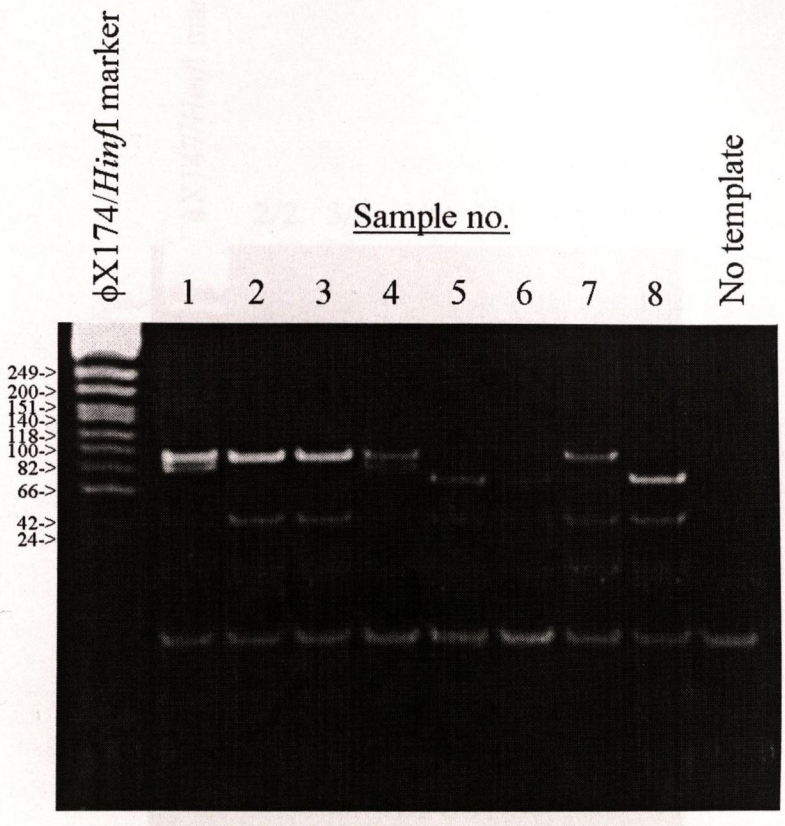

Figure 5.1.2: Endonuclease digested buffy coats DNA amplified for the Apo-E gene electrophoresed in 20% PAGE-TBE gel: *0.5µl of 1 in 10 diluted donor buffy coats were amplified using primers for a 227bp section of the Apo-E gene. The product was then digested with Cfo I, electrophoresed in a 20% PAGE-TBE gel, and visualised under UV light, as described in section 2.13.*

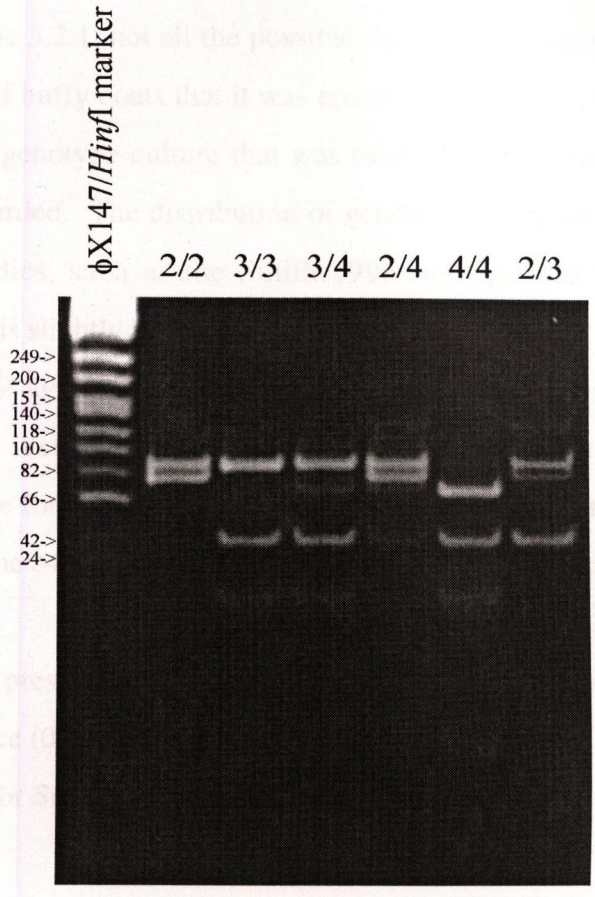

Figure 5.1.3: Endonuclease digested standard DNA amplified for the Apo-E gene electrophoresed in 20% PAGE-TBE gel: *0.5µl of known genotype DNA were amplified using primers for a 227bp section of the Apo-E gene. The product was then digested with Cfo I, electrophoresed in a 20% PAGE-TBE gel, and visualised under UV light, as described in section 2.13.*

5.2 DISTRIBUTION OF ε2, ε3 AND ε4 ALLELES IN DONOR BUFFY COATS.

The restriction pattern for each buffy coat PCR product was noted, and tabulated. As can be seen in Table 5.2.1, not all the possible Apo-E genotypes were encountered in the small number of buffy coats that it was possible to prepare primary cultures from. Also, the only 3/4 genotype culture that was established was subject to an infection and had to be discarded. The distribution of genotypes is somewhat different to that seen in larger studies, such as the Smith 1996 study of 308 Irish samples. 3/3 frequency, at 0.69, is slightly higher than would be expected (0.62 for Smith), as is the 2/3 genotype (0.19 against 0.09 for Smith). Also, the presence of the two 4/4 genotypes could be seen as unusual in such a small survey, but nevertheless this was quite useful to have for the comparison of Apo-E expression between genotypes, as any effects due to the ε4 allele would be larger with the homozygous form.

Allele frequency is presented in Table 5.2.2. The e3 wild-type allele is of exactly the expected prominence (0.80, 0.80 for Smith), whilst the ε2 allele (0.10, 0.05 for Smith) and ε4 (0.10, 0.15 for Smith) are of slightly altered frequency to that expected.

Table 5.2.1: Apo-E genotype frequency in donor buffy coats.

N	Genotype Frequency					
	2/2	2/3	2/4	3/3	3/4	4/4
26	0	0.19	0	0.69	0.04	0.08

All buffy coats received were examined by restriction fragment length polymorphism for Apo-E genotype, and the frequency of each genotype determined from the fragment pattern.

Table 5.2.2: Apo-E allele frequency in donor buffy coats.

N	Allele Frequency		
	ε2	ε3	ε4
52	0.10	0.80	0.10

All buffy coats received were examined by restriction fragment length polymorphism for Apo-E genotype, and the frequency of each Apo-E allele determined from the fragment pattern.

5.3 THE RELATIONSHIP BETWEEN APO-E GENOTYPE AND APO-E LEVELS IN PLASMA AND PRIMARY MONOCYTE CELL CULTURE MEDIUM.

The levels of Apo-E protein in serum are known to be partially a function of Apo-E genotype. Subsequent to separation of leukocytes from red blood cells by Histopaque density gradient (see section 2.4), a sample of the plasma was taken from the top of the tube and stored for later analysis. A volume of this plasma equivalent to 50μg of total protein was examined for Apo-E protein by SDS-PAGE electrophoresis and Western blotting. The autoradiogram in Figure 5.3.1 shows that there is a relatively consistent amount of Apo-E protein in these plasma samples. There is also quite an amount of cross reactivity with Human Serum Albumin.

The intensity of each Apo-E band was measured by scanning densitometry, and compared with the genotype for that sample. These results are presented in Table 5.3.2. The 3/4 and 4/4 genotypes are too infrequent for their associated data to be relevant, but in the case of the 2/3 genotype, a 50% increase in plasma Apo-E levels was observed. Primary monocyte cultures were prepared from each of these samples, and, after activation with PMA, were incubated with either IFN-β or IFN-γ for 5 days. The medium from these cells was examined for Apo-E by Western blotting, and the relevant autoradiogram sections presented in Figure 5.3.3. Here we can see visually that there is an almost identical suppression of Apo-E band intensity by IFN-β and IFN-γ.

These bands were assessed quantitatively using scanning densitometry, and the results presented in Table 5.3.4. The effects of IFN-β and IFN-γ are remarkably similar, with around 85% suppression of Apo-E secretion evident in the presence of 100U/mL of each cytokine. The Apo-E protein level is also almost identical between the two genotypes examined, with the 3/3 genotype only 5% less inhibited under the influence of 10U/mL of IFN-γ as the 4/4 genotype.

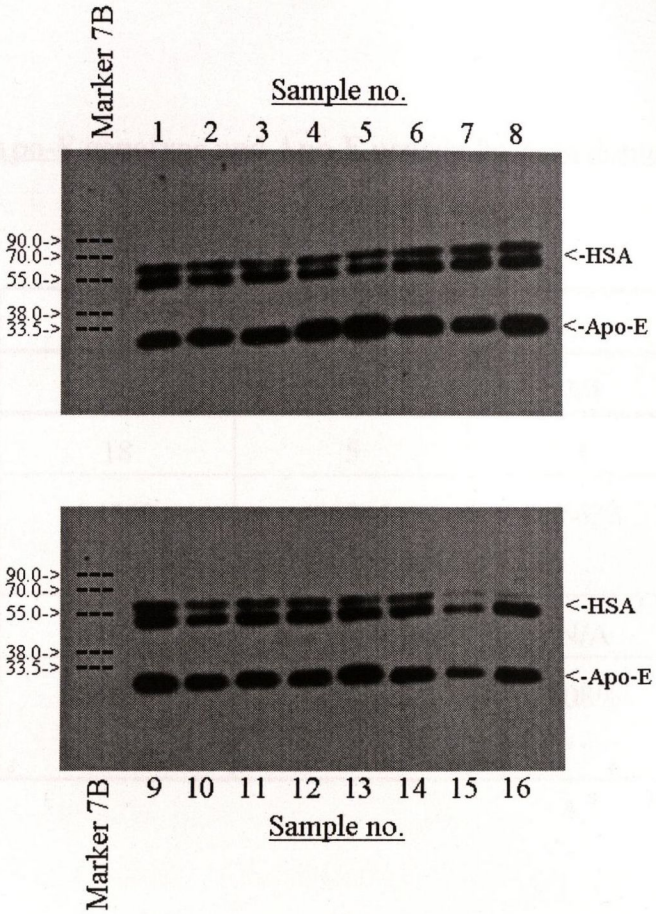

Figure 5.3.1: Western blotting of plasma from donor buffy coats for Apo-E: *Plasma was prepared from donor buffy coats, and a volume equal to 50µg of total protein examined for Apo-E using SDS-PAGE electrophoresis followed by Western blotting. Bands were visualised using chemiluminescent substrate, as described in section 2.11.*

Table 5.3.2: Apo-E genotype and Apo-E protein levels in donor buffy coats.

	Apo-E Genotype			
	3/3	**2/3**	**3/4**	**4/4**
N	18	5	1	2
Mean Apo-E intensity	24560	36845	26633	29774
SD	8371	5314	N/A	95
% Expression to 3/3	100%	150%	108%	121%

10μg of total protein from donor buffy coats was examined for Apo-E protein using SDS-PAGE electrophoresis followed by Western blotting. Apo-E signal density was examined by scanning densitometry, and the mean change in intensity for each sample expressed against the Apo-E genotype for those samples.

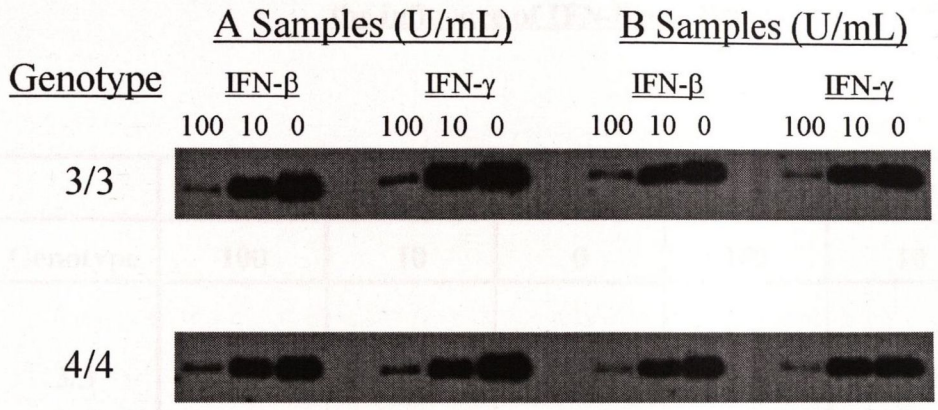

Figure 5.3.3: Western blotting of secreted protein from primary monocyte cultures for Apo-E: *10μg of concentrated secreted protein from 5 day, cytokine treated, primary monocyte cultures were examined for Apo-E using SDS-PAGE electrophoresis followed by Western blotting. Bands were visualised using chemiluminescent substrate, as described in section 2.11. Apo-E genotype was assessed using RFLP (see section 5.1).*

Table 5.3.4: Apo-E genotype and Apo-E secretion by primary monocytes under the influence of IFN-β and IFN-γ.

	IFN-β (U/mL)			IFN-γ (U/mL)		
Genotype	**100**	**10**	**0**	**100**	**10**	**0**
3/3 (n=2)	14%	75%	100%	14%	81%	100%
4/4 (n=2)	17%	75%	100%	13%	76%	100%

Secreted protein from 5 day IFN-β or IFN-γ treated primary monocyte cultures were examined for Apo-E protein using SDS-PAGE electrophoresis followed by Western blotting. Apo-E signal density was examined by scanning densitometry, and the mean intensity expressed as a percentage of the control intensity for each genotype.

CHAPTER 6

DISCUSSION

Modulation of Apo-E mRNA translation and of Apo-E protein have been reported in damaged central and peripheral nerves [Ignatius et al 1986]. Apo-E is involved in the repair process in peripheral nerve regeneration. In MS, demyelination involves the release of lipid components of myelin and Apo-E is the major lipoprotein transporting protein in the brain, as Apo-B is not found in the CSF. Demyelination involves inflammatory processes in which cytokines are modulatory factors. Interferon-β has been shown to ameliorate progression in MS by decreasing relapses but is expensive and not always successful.

The original hypothesis underlying this study was that the modulatory response to cytokines could be assessed in peripheral blood mononuclear cells from MS patients and controls. However, before undertaking studies on MS patients, it was decided to examine model systems in which cytokine effects could be investigated.

6.1 THE MODULATION OF APO-E mRNA AND PROTEIN IN ASTROCYTOMA CELL LINES BY CYTOKINES.

6.1.1 BACKGROUND.

The original purpose of this part of the study was to develop a model in which the modulation of Apo-E production in the astrocyte cell by cytokines could be investigated. The astrocyte is the second largest producer of Apo-E in the body [Elshourbagy et al 1985], and the major source of Apo-E in the brain. This Apo-E is believed to function in the provision of lipid and cholesterol to the neuron via the Apo-B/E (or LDL) receptor. In the case of an inflammatory MS attack, the increased levels of IFN-γ, TNF-α and IL-1α influence the astrocyte by inducing the production of cytotoxic substances. Apo-E levels in cerebrospinal fluid are known to drop during an MS attack [Rifai et al 1987], so it could be assumed that this drop is associated with the elevated levels of inflammatory cytokines affecting the astrocyte. There is currently no data for Apo-E levels in the CSF of patients taking IFN-β therapy.

Apo-E transcription was to be measured at the mRNA level using a DIG labelled DNA probe. mRNA for the cytoskeletal β-actin protein was used as an internal reference for Apo-E modulation by cytokines. Cellular and medium Apo-E protein levels were to be measured using Western blotting, and ^{35}S methionine labelling followed by immunoprecipitation where necessary.

6.1.2 SUITABILITY OF THE 1321N1 AND C6 ASTROCYTOMA CELL LINES AS A MODEL FOR APO-E PRODUCTION.

Studies in the transformed astrocytoma cell line CCFSTTG1 have shown that these astrocyte cultures can produce significant amounts of Apo-E protein [Krul and Tang 1992], and that the control mechanisms for Apo-E production in these cells closely resemble those found in the macrophage. Most experimentation in this area is still performed using primary astrocyte cells cultures, however, most commonly from a rodent source. There is no variation in Apo-E genotype in these, or indeed in most other mammals. This makes it very difficult to examine the production of Apo-E in cells of differing genotype, where the only possibility is a stable transfection with the required genotype. Due to the many levels of control seen in Apo-E production by the cell, a stable transfection is unlikely to be operating in this regard in a physiologically relevant manner. It was deemed beyond the scope of the project to extract primary astrocytes, and so the readily available 1321N1 and C6 astrocytoma cell lines were employed, both of which are quick growing and robust.

The cDNA used to construct probes for Apo-E mRNA were originally developed for studies on rat lipoproteins, and were known to work perfectly well with human cell lines such as HepG2, and CaCo-2 [Killalea 1997]. Sequence analysis of human and rat Apo-E mRNA using the EMBL database shows homology of at least 77%. Obviously there would be no problem with using these probes in conjunction with the C6 rat cell line.

6.1.3 THE EFFECTS OF INFLAMMATORY CYTOKINES ON THE TRANSCRIPTION OF APO-E mRNA IN ASTROCYTES.

Due to the low stability of Apo-E mRNA, it was deemed suitable to refer to the amounts measured as reflective of translation rates rather than merely of accumulation of the mRNA in the samples.

The Digoxigenin labelling kit from Boehringer Mannheim was employed for several reasons in place of the standard ^{32}P labelling. Firstly, it was a much more sensitive method of detection, which was thought would be important for later primary monocyte experiments where there would be very small cell numbers. Secondly, since it is a relatively non-toxic procedure, there would be no necessity for radiation badges or special waste disposal protocols. Thirdly, where many RNA samples were to be examined, the probes were reusable. Probes were found to be perfectly functional at the third or even fourth use, with only slight reductions in signal intensity. Often lower background levels were observed in these later blottings. Once prepared, the concentrated probe stocks were kept for up to the recommended one year at -20^{o}C. Again this appeared to have no detrimental effect on signal intensity or background on the autoradiograms.

The two different cDNA containing plasmids were slightly different in their performances. Since the Apo-E probe was not actually generated from an insert but a linearised plasmid (see Figure 3.1.1), there was a certain percentage of DIG labelled fragments that would not be complimentary to the Apo-E mRNA. This was not deemed to be a problem, as the fragments derived from a bacterial plasmid should not have any cross-reactivity with human mRNA. Nevertheless, the Apo-E probe appeared to suffer slightly more from speckled or cloudy backgrounds (as in Figure 3.1.1).

TNF-α and IL-1α both appear to inhibit the level of Apo-E mRNA in 1321N1 cells (see Figures 3.4b and 3.4c). They appear to have very similar activity in this regard,

with a drop of around 60% in each case at 1ng/mL. This is within the range of TNF-α used by Mazzone in monocyte/macrophage experiments. Infiltrating T-lymphocytes at the site of the MS lesion produce large amounts of TNF-α and IL-1α, and macrophages at the site may also have some influence. These substances would appear to signal to the astrocyte to cease exporting lipoprotein particles to the neuron through down-regulation of Apo-E, and thus limit axonal regeneration. The action of these cytokines is probably therefore localised to the nucleus, the ultimate site of action of most cytokines.

In transformed THP-1 monocyte cells, both TNF-α and IL-1α substantially increase the transcription of Apo-E mRNA [see Section 6.2.3]. This is the opposite of the case in 1321N1 cells. It would appear that the roles of the astrocyte and the macrophage in lipid recycling during the MS relapse and remission phases may not be as closely linked as was originally suspected, if the RNA data from the 1321N1 cells is indeed relevant. There may also be temporal events involved, where the snapshot taken at day five of a timecourse is not the most important sample. Earlier astrocyte or later monocyte/macrophage actions may be vital to the regeneration of neurons, but the number of samples required to prove this was prohibitive, so a five day run was used for all experiments.

IFN-γ has been shown to inhibit Apo-E secretion by THP-1 monocyte cells [Brand 1993]. In these cells the mRNA levels for Apo-E, and the cellular protein levels, remain almost unchanged at 100U/mL of IFN-γ, but there is still a drop in secretion of about 50%. There is no drop in Apo-E mRNA evident in 1321N1 cells at the same concentration (see Figure 3.4a). In this regard astrocytes would appear to be similar to macrophages, in that IFN-γ has little or no control at a transcriptional level, as judged by mRNA levels.

6.1.4 THE EFFECTS OF INFLAMMATORY CYTOKINES ON THE TRANSLATION OF APO-E mRNA IN ASTROCYTES.

Neither of the transformed cell lines examined, 1321N1 and C6, appeared to translate or secrete any Apo-E when examined by Western blotting followed by chemiluminescent substrate visualisation (see Figures 3.5.4b, 3.5.4c and 3.5.4d). When this method was used, 0.01μL of human plasma was seen to be sufficient to give a strong signal in less than one minute of exposure time to film. This volume would correspond to less than one nanogram of Apo-E protein, assuming average serum concentrations of about 50mg/mL were present in the donor blood.

The HepG2 secreted protein used to optimise the Western blotting procedure appears to give an excess of signal using chemiluminescent substrate, even with less than 1μL of raw medium (see Figure 3.5.4a). This would correspond to about 1/1000th of the total protein in any given cell culture medium sample, whilst even with concentration of the astrocyte samples, generally as much as 1/10th of the total protein present that was over 10000kDa would be used per well. DIG test blotting, however (see Figure 3.3.2), would seem to indicate that there is a comparable amount of Apo-E coding mRNA present in these cells HepG2 to 1321N1 astrocyte cells. This would suggest that even though there are similar levels of Apo-E mRNA in the two cell types, there is a massive difference in translation of this mRNA.

There may be some speculation as to whether these astrocyte cell lines are truly operating in a physiologically relevant environment. Macrophage Apo-E protein is known to be degraded in the absence of the free lipids necessary for lipoprotein assembly. There may be a similar control system present in the astrocyte, and perhaps the Sigma Serum Replacer II solution does not contain sufficient quantities to allow secretion of the completed lipoprotein complex. During the preparation phase of the astrocytes, there was generally quite vigorous growth in the presence of 5% FCS. This growth was observed (visually) to be severely restricted in the presence of serum replacer, but all cells remained adherent. This was somewhat important, as any

further growth in the wells would result in superconfluency, and affect the actions of the cytokines, and further remove some cells from the concentrations of lipid believed to be required for Apo-E secretion.

In later experiments using THP-1 and activated primary monocytes, there did not appear to be any Apo-E secretion problems associated with lack of FCS, so it seems most likely that the astrocyte cultures 1321N1 and C6 simply do not translate or secrete Apo-E protein.

In order to test this hypothesis, an ^{35}S labelling experiment was performed with the 1321N1 astrocyte cells. This could be done in the presence of FCS, as only *de novo* synthesis would be measured. FCS levels were maintained at 5% throughout this experiment. Some superconfluency was noted at the 4th and 5th days of culture, although the amount of incorporated ^{35}S labelled methionine, as measured by liquid scintillation counting, appeared to remain linear. This would show that almost all cells were still in contact with the medium and using the labelled methionine available. The total cellular extract protein stained after SDS-PAGE electrophoresis (see Figure 3.6.2) would indicate that there was very little change in total protein levels over the timecourse.

The total medium protein levels were quite low, with hardly any bands visible around the 33.5kDa marker where Apo-E would be expected (also Figure 3.6.2). Total excreted protein from astrocytes may always be low, and the measurements taken in Markwell protein estimations may be due mainly to albumin and supplement proteins in the provided medium and FCS.

The total precipitates are dominated by the heavy and light chain domains of the antibody used to bind Apo-E (see Figure 3.6.3). This would be expected if the precipitation is specific for Apo-E, as few other proteins are present and the Apo-E itself band should not be visible. There appeared to be a good amount of labelled proteins in the cellular extract when viewed with PPO scintillant, but no bands were

visible in the medium samples (see Figure 3.6.4). The medium samples may need to be concentrated in some way, but some bands would be expected in any case when there is so much labelled cellular protein.

There were no bands visible in the immunoprecipitant from the medium either (see Figure 3.6.5), but some were evident in the immunoprecipitated cellular extract. The heavier of these bands would appear to correspond to the larger bands found in the post-immunoprecipitate, showing that the system is not 100% monospecific and is also binding other proteins. There is one faint band visible at around the 33.5kDa marker, which may correspond to Apo-E in the cell. A second protein is nearly always seen in conjunction with Apo-E at a slightly lower molecular weight. This may be an earlier stage in the protein's production, before glycosylation has occurred. There is no evidence of this second protein in these cells. If this protein is indeed Apo-E, it is still at too low a concentration to be of any use in quantitative experimentation, particularly in conjunction with a scanning densitometer. Also, the complete lack of secreted Apo-E would render the 1321N1 cells useless as a model for lipid metabolism in the MS brain.

LDH assays on the 1321N1 experiments were satisfactory (see Table 3.7), with only the IFN-γ appearing to have any cytotoxic effects. Due to the lack of change in the Apo-E/β-actin ratios, it can be assumed this cytotoxicity was having no specific effect on Apo-E transcription.

6.1.5 METHODOLOGICAL CONSIDERATIONS.

When using the Serum Replacer (Sigma), the provided documentation suggests that it is possible to acclimatise cell line to grow and divide through gradual replacement of FCS with the Replacer fluid. This was attempted with the 1321N1 and C6 cell lines, but there was never any growth evident after reseeding, and indeed most cells refused to re-adhere to the plates. It was decided to grow cells to confluency in the presence of 5% FCS and then wash the cells clean of FCS containing medium using PBS.

After this, experiments could then proceed in the presence of Serum Replacer alone. What effect the maintenance of these cells in FCS free medium has on metabolism is unknown.

The generally accepted protein concentration to be used per well in a mini-gel set up is 50µg. Attempts were made to use more than this when looking for Apo-E protein in the astrocytoma lines, without success. This would often lead to deformation of lanes, and would probably result in protein masking, where other proteins in the solution can block the epitopes recognised by the antibody being used.

It was quite common for the Dako anti-Apo-E antiserum to cross-react with albumin in human serum, creating a doublet at around 66kDa (see Figure 3.5.3a etc.). It is unlikely that any epitope of Apo-E could survive bound to HSA after boiling in the presence of mercaptoethanol, so it is possibly the case that the HSA can bind the antibody non-specifically. This could be expected as albumin is used by the blood as a carrier for many proteins. Slightly more puzzling was the presence of two bands in the marker lane. These would appear to be pyruvate kinase (70kDa) and fructose-6-phosphate kinase (90kDa), which are listed in the Sigma Prestained Marker 7B data sheet. A blot was tested for cross-reactivity with the secondary antibody, with no success, meaning that the Dako antibody must not be entirely monospecific. A monoclonal antibody was deemed to be unnecessary as the proteins were not of similar sizes to Apo-E.

The ^{35}S labelling method employed was based on that of Williams and Dawson [Willams 1986]. This paper suggests using 200µCi/mL for avian skeletal muscle cells, avian or monkey fibroblasts, and aortic smooth muscle cells. No data exists for immunoprecipitation of Apo-E from astrocytes. Since no bands were obtained after PPO visualisation of the labelled proteins in SDS-PAGE gels, it was deemed impossible to excise any bands for the more sensitive direct scintillation method. If this were possible, Williams and Dawson claim that Apo-E at any level above 0.02%

of total protein can be detected. It can be concluded that whatever amounts are translated by 1321N1 cells, it is much lower than this 0.02% level.

6.2 THE MODULATION OF APO-E mRNA AND PROTEIN IN PRIMARY AND TRANSFORMED HUMAN MONOCYTES BY CYTOKINES.

6.2.1 BACKGROUND.

The macrophage is a major source of Apo-E protein in humans. It is believed that the macrophage may facilitate the regeneration of neuronal cells through provision of lipid and cholesterol, particularly for cell membrane construction. The objective here was to reproduce some of the conditions found in an inflammatory MS attack, where infiltrating monocytes are activated to become macrophages, and their production of Apo-E is modulated by inflammatory and anti-inflammatory cytokines.

6.2.2 SUITABILITY OF THE THP-1 MONOCYTE CELL LINE AND PRIMARY MONOCYTES AS A MODEL FOR APO-E PRODUCTION.

Inactivated, or "resting" monocytes do not produce significant quantities of Apo-E mRNA or protein. This only occurs during and after differentiation to become a macrophage. The THP-1 human monocytic cell line has been widely employed for experimentation in a variety of roles, including cytokine production, second messenger system signaling, and Apo-E production. It is normally a suspension cell line, and only adheres upon activation with PMA, IFN-γ, UV light, or lipopolysaccharide. It is not, however, 100% identical to a blood resident monocyte, as it requires 10 times more PMA to bring about activation (10^{-7}M instead of 10^{-8}M). The cell line is known to respond to and produce the cytokines involved in the inflammatory response.

Primary blood monocytes are more difficult to prepare, and, since they do not divide, also come in fixed numbers. They are, however, more representative of true physiological function. Buffy coat samples may be heterogenous for Apo-E, unlike the THP-1 line which is ε3/ε3.

6.2.3 THE EFFECTS OF INFLAMMATORY CYTOKINES ON THE TRANSCRIPTION OF APO-E mRNA IN THP-1 MONOCYTES.

The RIBO-Green assay proved to be extremely sensitive for even the tiny amounts of RNA extracted from primary monocyte preparations. When converted to cDNA, the TaqMan PCR process appeared to work perfectly. Control tubes, containing no template, normally had a C_t of 40, meaning that the amount of double stranded DNA was insignificant in comparison to those tubes with sample. Some controls had a C_t of 5 to 6 units larger than the template containing tubes, but even in these cases, the amount of fluorescence seen would be 32 (2^5) to 64 (2^6) times less. This would correspond to a maximum of 3% of the fluorescence deriving from primer dimers or other sources, and was ignored.

Three of the four inflammatory cytokines examined (TNF-α, IL-1α and TGF-β) appeared to greatly promote Apo-E mRNA transcription [see Tables 4.3.3a, 4.3.3b and 4.3.3c respectively] in the THP-1 cells. The TNF-α at 1ng/mL gave a 900% increase, with no associated cytotoxicity [see Table 4.5.1(I)]. This promotion was dose-dependent. Similarly, IL-1α gave an over 500% increase, again with no associated LDH increase. In the case of TGF-β, however, there was an increase of 500% in Apo-E mRNA at 10^{-2} ng/mL, but an almost 100% increase in detected LDH activity. This would indicate that for this cytokine, at this concentration, there is an amount of cytotoxic activity. There appears to be a quite significant rise in the C_t for G6PDH in THP-1 cells with TGF-β, signifying a large drop in G6PDH expression. This is most likely related to the general drop in RNA and protein production seen under cytotoxic conditions, as G6PDH should not be specifically targeted by TGF-β.

Since, in the environment of an MS attack, there is an overall drop in secreted Apo-E protein detectable in the CSF, it may be said that the influence of IFN-γ is predominant, as this cytokine appears to bring about a drop in Apo-E secreted protein levels in primary astrocytes [Oropeza et al 1987]. In the THP-1 cells examined, IFN-γ at 100U/mL caused a drop of only 17% in Apo-E mRNA [see Table 4.3.3d]. IFN-β-1b at the same concentration caused only an 11% drop. In combination, these two cytokines appear to operate synergistically, with a drop of 24% in Apo-E transcription at 100U/mL of each. These figures may not be entirely important to the functioning of these cytokines in regulation of Apo-E expression, as transcription appears to be a less important aspect in control of Apo-E protein secretion.

There was an almost 50% rise in LDH activity in the medium from THP-1 cells after incubation with IFN-γ for 5 days [see Table 4.5.1(II)]. However, there was almost no change in the C_t for the G6PDH amplicon [see table 4.3.3d(II)], so the relative Apo-E levels would appear to be significant. No cytotoxicity was observed with IFN-β-1b.

6.2.4 THE EFFECTS OF CYTOKINES ON THE TRANSLATION OF APO-E mRNA TO PROTEIN IN MONOCYTES.

After setting up the THP-1 cell line and ensuring Apo-E production has been preserved (see Figure 4.1), Apo-E production in the presence of inflammatory cytokines was examined. IFN-γ was shown to affect Apo-E translation as expected (see Figure 4.1.1a), giving suppression at 100U/mL. The expected doublet was also visible, with the lower molecular weight unsialylated protein running slightly faster in the gel. The inhibition of Apo-E cellular protein by IFN-γ appeared to be at its greatest at day 5.

The other inflammatory cytokines did not appear to have any profound affect of cellular Apo-E protein levels [see Figures 4.1.1b, 4.1.1c and 4.1.1d]. A small drop seemed to be observed with higher amounts of TNF-α (but interestingly not at the top concentration), and with IL-α or TGF-β at medium concentration ranges. This

repression did not seem to be dose dependent, which makes its significance quite unclear. There is a possibility that at moderate levels of these cytokines the post-transcriptional repression is at it's peak, and only later at higher concentrations is the Apo-E mRNA level upregulated sufficiently to negate this. The stimulation of mRNA levels seems to be dose-dependent, whereas the inhibition of translation does not.

This was in quite stark contrast to the results observed from the RT-PCR experiments, where all three cytokines caused a massive increase in Apo-E mRNA transcription. There are therefore at least two levels of control of Apo-E protein production under the influence of these cytokines, as the increased mRNA does not cause any discernable increase in protein accumulation in the cell or medium.

In primary monocyte cells, cellular levels of Apo-E were more vigorously inhibited by IFN-γ at 100U/mL than in the THP-1 cells [see Figure 4.2.1a]. Also, the maximum inhibition appeared to occur at day three of the time course, rather than day five for the transformed line. This would indicate greater sensitivity in primary cells than in the THP-1 cell line. Again the alternative cellular form of the protein was visible. Whilst the biochemistry of the THP-1 cell line appears to be grossly similar to the primary monocyte, it is clear that for good, physiologically relevant data, only the latter is indeed suitable.

IFN-β appeared to have a slight inhibitory effect on cellular Apo-E levels [see Figure 4.2.1]. This slight inhibition was still evident in the presence of IFN-γ, so generally both cytokines appeared to have a similar influence on Apo-E cellular protein levels as they did on Apo-E mRNA levels, i.e. slightly inhibitory. In the case of IFN-β then, perhaps there is no extra control structure between Apo-E transcription and translation, but the drop in cellular Apo-E levels seen here, and in Figure 4.1.1a, cannot be fully accounted for by the mere 17% drop in Apo-E mRNA seem in Table 4.3.3d.

6.2.5 THE EFFECTS OF CYTOKINES ON THE SECRETION OF APO-E PROTEIN IN PRIMARY MONOCYTES.

When secreted protein was examined in the THP-1 cell line, there was a significant drop in Apo-E protein in the medium observed at day 5 with 100U/mL of IFN-γ [see Figure 4.1.2.a]. This would appear to correspond with the drop in cellular Apo-E levels and Apo-E mRNA levels, but is much more inhibited than would be expected. None of the other inflammatory cytokines examined [see Figures 4.1.2b, 4.1.2c and 4.1.2d] appeared to have any major effect on secreted Apo-E levels. The intriguing point here is that those cytokines which appear to increase Apo-E mRNA translation appear to have no significant end effect on Apo-E secretion, whereas IFN-γ, which inhibits Apo-E mRNA slightly, appears to have its effects amplified.

In primary monocytes, the secretion of Apo-E is inhibited by both IFN-β and IFN-γ, with IFN-γ appearing to be the slightly stronger inhibitory factor [see Figure 4.2.2b]. Interestingly, at 1U/mL of IFN-γ there seems to be an increase in Apo-E secretion, and this increase occurs at every concentration of IFN-β. Above 1ng/mL, IFN-γ causes severe downregulation, especially in the presence of any IFN-β. Obviously, there is no antagonistic action, with regard to Apo-E, by IFN-β and IFN-γ. In fact both cytokines appear to act synergistically. It must be assumed from this that the reduced lesion load and neurological damage observed in MS patients undergoing IFN-β treatment is not due to increased remodeling of the neuron under the influence of Apo-E. The drop in Apo-E levels seen with IFN-β here could be imagined to be a severe setback to neuronal repair.

In light of this, the macrophage may not be the most important cell for providing lipid and cholesterol, with the astrocyte performing a more important role. Control structures in the astrocyte may be significantly different, as inferred from the results examined in Section 6.1. There, astrocyte Apo-E mRNA levels were seen to drop with TNF-α and IL-1α, rather than increase as they do in the monocyte. A protein producing model of this modulation would be very useful, as if IFN-β were to inhibit

that (presumably more physiologically important) inhibition, there would be a more plausible explanation for IFN-β's effects in this area. IFN-β's general control over macrophage proliferation and cytotoxic activity during the MS relapse may be more important for sufferers than control over neuronal regeneration during remission.

6.2.6 METHODOLOGICAL CONSIDERATIONS.

Experiments using monocyte cultures can be difficult to reproduce, as there may be a great deal of variation between donor samples, not just in the Apo-E gene. In some cases, there is obviously some form of infection being combated by the leukocyte population, and there is an increase in the numbers of leukocytes present. Whether these cells are activated to some extent under these conditions is unknown. Some buffy coats received had large numbers of trypan blue-porous leukocytes, meaning the cells had died during storage at the blood bank. These cells were discarded, but buffy coat and plasma samples were still retained for genotyping and Apo-E quantification, respectively.

After seeding, monocytes begin to adhere and flatten to the basal surface, in this case the treated plastic dish. Quite often, where total cell numbers were low, the aspirated leukocyte suspension was used for a second round of seedings. Adherent monocyte yield from this seeding was generally good, although cells were not as numerous as the first instance. After adhering, monocytes eventually produce keratin as a matrix factor. This cannot be digested by the trypsin normally used to lift adherent cells. Therefore monocyte cell numbers were unknown, and were assumed to be constant between wells.

The cytokines chosen for investigation reflected the number of cells available. The more abundant THP-1 cells were used with TNF-α, IL-1α and TGF-β, as well as IFN-γ. When cell numbers were limiting, as with the primary monocyte experiments, only the perceived most relevant cytokines IFN-β and IFN-γ were examined. This was necessary due to the need to do all experiments in triplicate, and with a range of

cytokine concentrations, using samples at least large enough to give acceptable protein levels for Western blotting.

In order to ensure that cytokine influences were not due to cytotoxic effects, LDH assays were carried out on all medium samples. However, in the case of the primary monocyte it was also decided to employ an Eosin Y/Methylene Blue staining technique. On the fifth day after activation, cells that had been treated with 100U/mL of IFN-γ were lifted from their well, using a cell scraper, and resuspended in 100μL of PBS. The cells were dried onto a microscope slide and the Eosin Y and Methylene Blue stains applied sequentially. Slides were then visualised at 100X magnification and photographed, this time at 32X magnification. As can be seen in Appendix 3, the cells are mostly still intact, and have taken up little or no Methylene Blue into their cytosol. They also have large globular nuclei, indicative of cells that are not undergoing apoptosis. Thus we can conclude that these cells are still mostly viable at the fifth day of incubation.

The Perkin-Elmer TaqMan thermal cycler is a much more efficient machine than the standard laboratory type. The heated lid and extremely fast ramping times between PCR stages means that many seconds may be cut from each cycle. As a consequence, the amplified DNA products are exposed for less time to high temperatures and should be better preserved. Also, for the same reason, the Taq polymerase enzyme used should be more stable, and give a higher yield with greater accuracy.

Raw results obtained from the GeneAmp cycler [see Appendix 1] were quite abundant, so a special program was written to calculate the relative amount of expression based on the C_t values of the triplicate cDNA samples examined [see Appendix 5]. This was found to be much simpler than setting up an Excel worksheet to do the same task.

6.3 THE EFFECTS OF APO-E GENOTYPE ON MONOCYTE PRODUCTION OF APO-E PROTEIN.

6.3.1 BACKGROUND.

As mentioned in Section 1.5.5, Apo-E genotype has been epidemiologically associated with several neurological disorders. The modulation of Apo-E secretion by several cytokines has also been shown to be partly a function of the phenotype of the differentiating monocyte [Duan and Mazzone 1995]. Although Apo-E levels in serum and CSF are mainly thought to be controlled through clearance rates via the Apo-B/E receptor, and the different genotypes to have different binding affinities, it was considered worthwhile to examine any effects Apo-E genotype may have on the protein secretion itself. As the macrophage also expresses the Apo-B/E receptor, there may also be some role for re-uptake rates to play in a single cell-type scenario.

The easiest way to ascertain Apo-E genotype is through examination of blood samples, in this case by PCR. This should give some data as to the general distribution of genotypes in the samples. Production of Apo-E protein by primary monocyte cultures of differing genotypes could then be examined.

6.3.2 THE DISTRIBUTION OF APO-E ALLELES IN DONOR BUFFY COATS.

The modification of the PCR protocol to suit the leukocyte-enriched buffy coats was found to only require a slight increase in $MgCl_2$ concentration to 25mM [see Figure 5.1.2]. This compares with the 10mM used for regular blood samples. The reason for this increase is probably due to the magnesium binding properties of DNA, and there should be much more target DNA in a buffy coat preparation. The DNA reference standards provided by Dr.Wenham also appeared to amplify perfectly. Primer dimers were not such a problem here as they were in the case of the SYBR-Green RT-PCR, but they were apparent in some TBE-PAGE gels as the smallest band visible.

Table 5.2.2 indicates a reasonably representative mix of Apo-E alleles were found in the 26 buffy coats examined, even though the sample size was relatively small. The number of blood samples genotyped was restricted by the need to prepare a primary culture from each buffy coat received, as the data gathered from genotyping alone was irrelevant without the accompanying Apo-E modulation data for that sample. 80% of Apo-E alleles in the Irish population were ε3 in another study [Smith 1996]. The counts for the ε2 allele here are a little high, and that for ε4 a little low. More ε4 would be normal for a population such as Ireland's, where there is quite a high risk of late onset type Alzheimer's disease. However, even though the overall ε4 allele frequency is low, Table 5.2.1 would indicate a higher than expected incidence of the ε4/ε4 genotype than expected. It is especially unusual that this frequency would be higher than that of the ε3/ε4 genotype. This was most likely simply an anomaly due to the small sample number used. Only one example of the ε3/ε4 genotype was encountered, and this proved to be infected and had to be discarded. In any event, the two ε4/ε4 genotypes were deemed to be of even greater value, as any allele specific effects on Apo-E production should be increased in these cells.

6.3.3 THE RELATIONSHIP BETWEEN APO-E GENOTYPE AND APO-E PRODUCTION BY CELLS UNDER INFLAMMATORY AND/OR ANTI-INFLAMMATORY STIMULI.

The results presented in Table 5.3.2 would indicate that there is some change in the Apo-E protein levels in plasma samples with Apo-E genotype. Since these are not fasted patient samples, not all of the variation seen is due entirely to genotypic effects. However, the samples assessed would appear to agree with the expected findings where the ε2 allele is associated with higher plasma Apo-E. This is due to the reduced affinity of the Apo-B/E receptor for the ε2 form (<1% of normal), resulting in accumulation in plasma.

Under the more controlled environment of Apo-E production solely by monocytes, Apo-E secretion into medium is inhibited, as expected, by both IFN-β and IFN-γ [see

Figure 5.3.3]. The inhibition seen would appear to be almost identical for the two cytokines. However, when we compare the different genotypes, the ε3/ε3 and ε4/ε4 monocytes appear to respond almost identically to these cytokines. Densitometry readings [see Table 5.3.4] show around a 25% drop in Apo-E secretion with both genotypes when either cytokine is at 10U/mL. This drops to about 15% of normal secretion when either cytokine is at 100U/mL.

These results would indicate that control processes triggered by IFN-β and IFN-γ do not operate at the level of cytokines such as TGF-β, where there is a steep increase in Apo-E mRNA but not protein. The drop in Apo-E mRNA for IFN-β and IFN-γ may be more related to general downregulation of genes in the cell, and the modulation of secretion a more specific event.

The original proposition was at this point to move the experiment into a clinical phase, with the utilisation of blood from MS patients, both in receipt and not in receipt of IFN-b therapy. The Apo-E levels in the blood of these patients could be assessed, along with the genotype for each patient. The scale, however, would make the removal of most of the parameters used with the buffy coat experiments necessary. The average buffy coat is about 50mL, and corresponds to one unit of blood, or 500mL. This was usually found to be sufficient for about fifty 2.5cm diameter wells of primary monocytes. The average ampoule contains only 6mL of blood, and would be the most one could expect to take from an MS patient. This would mean less than one normal well of adherent monocytes per patient, so instead a 96 well plate would have to be used. RT-PCR and secreted protein measurement should be no problem with only 100μL of monocyte suspension instead of the usual 1mL, but cellular protein would have to be ignored.

6.3.4 METHODOLOGICAL CONSIDERATIONS.

Attempts were made to use the products from the RT-PCR experiments in Section 4 in genotyping, which would have removed the need for a separate amplification. Unfortunately, the CfoI restriction endonuclease did not appear to function correctly in the presence of the SYBR-Green dye, and the resulting PAGE-TBE gels only contained the 227bp product, together with several smears of unidentified DNA, It may have been possible to precipitate the DNA at this stage, but a separate RFLP procedure was eventually deemed simpler.

Leukocyte enriched buffy coats are generally prepared by centrifugation and screening for viruses performed on the day of blood collection. This process generally takes 24 hours, so there may be some drop in viable cell numbers due to this delay. Activation can also occur during this storage period. This would not be such a problem except for presence of T-lymphocytes in the mix. These cells start to produce large amounts of several cytokines when activated, which can be cytotoxic, and can sometimes also destroy the monocytes directly. On other occasions storage conditions can cause autohaemolysis of the remaining red blood corpuscles. This can cause activation of cells as they begin to treat the proteins released as foreign. Thus there are several ways in which the cells may be activated prior to commencement of the experiment, causing altered levels of monocyte differentiation in different donor samples.

CONCLUSIONS

Apo-E levels in the brain, and the genotypic dependent affinity of the neuronal LDL receptor to bind them, are crucial to repair and remodeling of neurons after neurological attack, such as the MS relapse [Ignatius 1986] . The inflammatory cytokines (IFN-γ, TNF-α, IL-1α) that are elevated during the MS relapse exert an influence on the important astrocytic production of Apo-E. We have shown here how they can influence Apo-E mRNA levels, but were unable to gather data for protein. Further studies, using other transformed cell lines or, preferably, primary astrocyte cultures, could give us many details concerning the secretion of Apo-E protein during the relapse, and also determine if the MS relieving drug, IFN-β, acts directly to counterbalance any inflammatory influence. In the infiltrating monocyte/macrophage, which can cross the damaged blood brain barrier during the relapse, we have shown Apo-E mRNA levels are boosted by TNF-α, IL-1α and TGF-β, but this does not translate into elevated levels of protein. Both the inflammatory IFN-γ and the anti-inflammatory IFN-β appear to have similar inhibitory effects on Apo-E secretion in these cells, also mostly not at a level of mRNA control. The Apo-E genotype does not appear to influence the effect of cytokines in this regard. The importance of Apo-E regulation during MS remission may be of more importance than during the relapse, and this coupled with the general immunosuppressive effects of IFN-β therapy may explain most of it effects.

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APPENDICES

APPENDIX 1: SAMPLE TAQMAN PCR RESULTS SHEET.

File:	21.6.00.rslts
User:	Administrator
Plate Type:	5700 Quantitation
Threshold:	0.500

Stage	Temperature	Time	Repeat	Ramp Time	Auto Increment
1	50.0 C	2:00	1	Auto	Auto Increment
2	95.0 C	10:00	1	Auto	Auto Increment
3	95.0 C	0:30	40	Auto	Auto Increment
	63.0 C	0:30		Auto	
	70.0 C	1:30		Auto	
4	70.0 C	10:00	1	Auto	Auto Increment
5			0		Auto Increment

Primer/Probe	Name	Task	Reporter	Quencher
ApoE	ApoE(E23,E31)		SYBR	SYBR
G6PD	G6PDH		SYBR	SYBR

Well	Type	Name	Primer/Probe	Ct
A5	UNKN	D1	G6PD	30.99
A6	UNKN	D1	G6PD	30.61
A7	UNKN	D1	G6PD	30.41
A8	UNKN	D1	G6PD	30.67
B5	UNKN	D1	G6PD	31.07
B6	UNKN	D1	G6PD	30.06
B7	UNKN	D1	G6PD	40.00
B8	UNKN	D1	G6PD	30.36
C5	UNKN	D1	G6PD	30.10
C6	UNKN	D1	G6PD	35.22
C7	UNKN	D1	G6PD	30.15
C8	UNKN	D1	G6PD	30.99
D5	UNKN	D1	G6PD	40.00
D6	UNKN	D1	G6PD	30.58
D7	UNKN	D1	G6PD	30.22
D8	UNKN	D1	G6PD	31.06

APPENDIX 2: SAMPLE TAQMAN CALCULATIONS.

(For THP-1 cells, control wells, see 4.3.3d)

C_t for Apo-E:	30.11	30.96	30.58
C_t for G6PDH:	31.54	31.89	31.74
$2^{Ct\ for\ Apo\text{-}E}$:	$2.32x10^9$	$2.09x10^9$	$1.61x10^9$
$2^{Ct\ for\ G6PDH}$:	$3.12x10^9$	$3.98x10^9$	$3.58x10^9$

Mean of $2^{Ct\ for\ Apo\text{-}E}$: $1.62x10^9$
Mean of $2^{Ct\ for\ G6PDH}$: $3.56x10^9$

To calculate x is in $2^x=1.62x10^9$

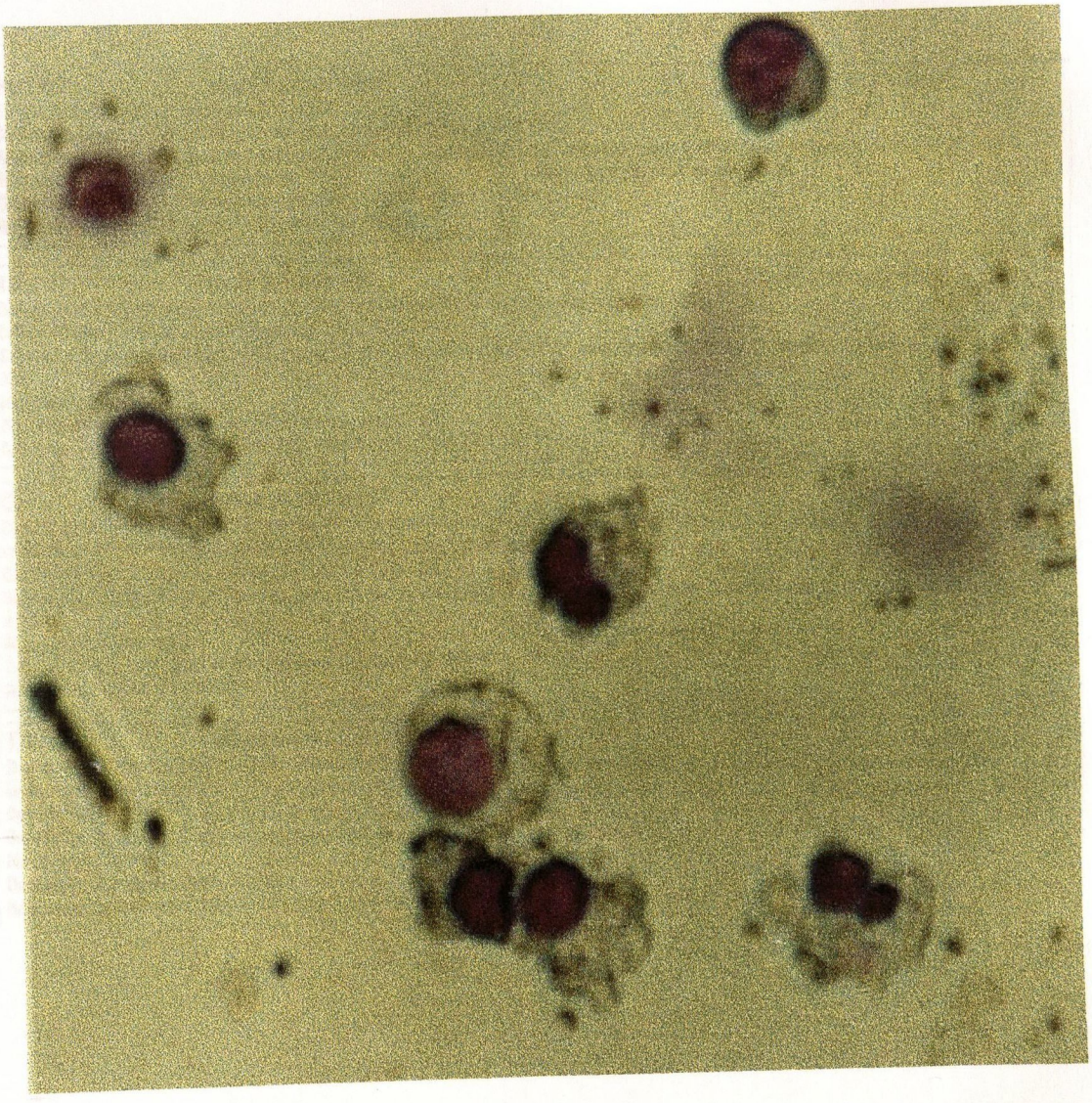
$$\frac{\text{Ln } 2^{Ct\ for\ Apo\text{-}E}}{\text{Ln } 2} = 30.59$$

$$\frac{\text{Ln } 2^{Ct\ for\ G6PDH}}{\text{Ln } 2} = 31.73$$

To translate into ratio:

$2^{(Ct\ for\ Apo\text{-}E)-(Ct\ for\ G6PDH)} = 2.20$

APPENDIX 3: EOSIN Y/METHYLENE BLUE STAIN ON PRIMARY MONOCYTES.



(X130 magnification)

APPENDIX 4: ECACC CATALOGUE ENTRIES FOR CELL LINES.

Hep G2 **ECACC No:** 85011430 **Human hepatocyte carcinoma**

Established from 15-year-old male Caucasian.
Culture Medium: EMEM (EBSS) + 2mM glutamine + 1% NEAA + 10% FBS
Subculture Routine: Split confluent cultures 1:3 to 1:6 ie seeding at 2-3x10^4 cells/cm^2 using 0.25% trypsin or trypsin/EDTA; 5% CO_2; 37°C
Morphology: Epithelial **Passage no:** 90 **Karyology:** 2n = 46; modal No. 55
Depositor: Professor B Knowles, Wistar Institute, Philadelphia
Reference: Nature 1979;282:615; Science 1980;209:497

1321N1 **ECACC No:** 86030402 **Human brain astrocytoma**

Derived from a human brain astrocytoma. The cell line has been mycoplasma eradicated using MRA.
Culture Medium: DMEM + 2mM glutamine + 10% FBS
Subculture Routine: Split confluent cultures 1:2 to 1:6 ie seeding at 2-4x10^4 cells/cm^2 using 0.25% trypsin or trypsin/EDTA; 5% CO_2; 37°C
Morphology: Glial
Depositor: Mr M Shaw, Bioscience, ICI Pharmaceuticals Ltd
Reference: Acta Path Scand 1972;80:267; Proc Nat Acad Sci, USA 1977;74:4816

C6 **ECACC No:** 85040101 **Rat glial tumour** RCD

Derived from a rat glial tumour induced by N-nitrosomethylurea. The cells produce S-100 protein which is found in the neural tissue of vertebrates. The syntheis of S-100 is dependent on the level of confluence.
Culture Medium: Hams F12 + 2mM glutamine + 10% FBS
Subculture Routine: Split confluent cultures 1:3 to 1:6 ie seeding at 1-3x10^5 cells/cm^2 using 0.25% trypsin or trypsin/EDTA; 5% CO_2; 37°C
Morphology: Fibroblast **Karyology:** 2n = 42
Depositor: Dr T J Hill, Dept of Microbiology, University of Bristol
Reference: Science 1968;161:370

THP 1 **ECACC No:** 88081201 **Human monocyte**

Derived from the peripheral blood of a 1-year-old boy with acute monocytic leukaemia. Cells do not express surface and cytoplasmic immunoglobulins. The cells are phagocytic and differentiate into macrophage-like cells.
Culture Medium: RPMI 1640 + 2mM glutamine + 20µM 2ME + 10% FBS
Subculture Routine: Maintain cultures between 2-9x10^5 cells/ml; 5% CO_2; 37°C
Morphology: Monocyte
Depositor: Dr J Clarke, AVRI, Pirbright
Reference: Int J Cancer 1980;26:171; Cancer Res 1982;42:1530; J Immunol 1983;131:1882

APPENDIX 5: BASIC PROGRAM FOR CALCULATING APO-E/G6PDH RATIOS FROM TAQMAN RT-PCR

```
10 CLEAR
20 PRINT "INPUT TARGET GENE CTS"
30 INPUT T1
40 INPUT T2
50 INPUT T3
60 PRINT "INPUT HOUSEKEEPING GENE CTS"
70 INPUT H1
80 INPUT H2
90 INPUT H3
100 LET MT = (2^T1+2^T2+2^T3)/3
110 LET MH = (2^H1+2^H2+2^H3)/3
120 LET T = LOG(MT)/LOG(2)
130 LET H = LOG(MH)/LOG(2)
140 LET X = 2^(H-T)
150 PRINT "2^(HOUSEKEEPING - APOE)=";
160 PRINT X
170 GOTO 20
1000 END
```

APPENDIX 6: SCANNING DENSITOMETRY VALUES FOR WESTERN BLOTS IN CHAPTER 4.

THP-1 cellular Apo-E.

[Cytokine]→	+++++	++++	+++	++	+	0
TNF-α	112587	66629	110615	126512	112940	163691
IL-1α	99035	104325	102449	86814	52958	134222
TGF-β	172785	136665	113442	98121	134657	154039

THP-1 secreted Apo-E.

[Cytokine]→	+++++	++++	+++	++	+	0
TNF-α	567385	435528	455093	434135	429758	534211
IL-1α	232736	226879	227568	256871	252338	246167
TGF-β	248811	233488	246065	232301	239218	253106

Primary monocyte cellular Apo-E.

IFN-γ→ IFN-β↓	+++	++	+	0
+++	25692	33382	30227	28483
++	22055	24644	58358	40216
+	27063	23178	33441	39897
0	32952	50300	41082	42175

Primary monocyte secreted Apo-E.

IFN-γ→ IFN-β↓	+++	++	+	0
+++	29573	66602	91843	75786
++	38902	110811	134146	116992
+	30283	104634	112858	112407
0	91383	121681	157043	127673

APPENDIX 7: RELATIVE DENSITOMETRY VALUES FOR WESTERN BLOTS IN CHAPTER 4.

THP-1 cellular Apo-E.

[Cytokine]→	+++++	++++	+++	++	+	0
TNF-α	69%	41%	68%	77%	69%	100%
IL-1α	74%	78%	76%	65%	40%	100%
TGF-β	112%	89%	74%	64%	87%	100%

THP-1 secreted Apo-E.

[Cytokine]→	+++++	++++	+++	++	+	0
TNF-α	106%	82%	85%	81%	80%	100%
IL-1α	95%	92%	92%	104%	103%	100%
TGF-β	98%	92%	97%	92%	95%	100%

Primary monocyte cellular Apo-E.

IFN-γ→ IFN-β↓	+++	++	+	0
+++	61%	79%	72%	68%
++	52%	58%	138%	95%
+	64%	55%	79%	95%
0	78%	119%	97%	100%

Primary monocyte secreted Apo-E.

IFN-γ→ IFN-β↓	+++	++	+	0
+++	23%	52%	72%	59%
++	30%	87%	105%	92%
+	24%	82%	88%	88%
0	72%	95%	123%	100%

APPENDIX 8: RAW C_t VALUES FOR TAQMAN RT-PCR IN CHAPTER 4.

THP-1 with TNF-α

ng/mL	1	0.1	0.01	0.001	0.0001	0
Apo-E	25.33	25.33	25.02	25.47	24.79	25.73
	24.85	24.80	25.56	25.23	25.18	26.49
	25.57	24.91	25.27	24.90	25.67	26.13
G6PDH	30.56	29.13	28.77	27.90	27.54	28.12
	30.92	29.35	29.40	28.69	27.91	27.62
	30.16	29.05	28.50	28.35	27.29	28.14

THP-1 with IL-1α

ng/mL	1	0.1	0.01	0.001	0.0001	0
Apo-E	25.82	26.72	26.42	25.38	25.86	25.70
	25.48	26.66	25.66	24.82	25.82	26.12
	26.02	26.30	25.95	25.05	26.49	26.48
G6PDH	29.80	30.88	29.04	27.76	27.21	27.30
	30.13	30.74	28.49	28.10	27.56	27.37
	29.99	31.43	28.20	27.76	28.14	28.02

THP-1 with TGF-β

ng/mL	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	0
Apo-E	32.14	31.57	30.88	31.08	29.23	30.71
	31.95	31.47	30.65	31.42	29.49	30.09
	32.28	30.97	31.18	31.43	29.76	30.71
G6PDH	35.13	34.16	33.88	32.96	31.16	31.24
	35.44	33.63	33.34	33.85	31.36	30.49
	35.09	34.57	33.08	33.14	30.57	31.29

THP-1 with IFN-β/IFN-γ

	IFNγ(U/ml)→ / IFNβ(U/mL)↓	100	10	0
Apo-E	100	31.05 30.47 30.81	30.94 30.51 30.25	30.81 30.25 31.10
	10	31.40 31.13 29.89	31.64 30.84 30.77	31.56 30.40 30.34
	0	20.84 30.50 30.11	31.20 30.72 30.96	30.43 30.41 30.58
	IFNγ(U/ml)→ / IFNβ(U/mL)↓	100	10	0
G6PDH	100	31.51 31.48 31.34	31.93 30.91 30.92	31.21 31.50 31.57
	10	32.28 31.66 31.90	32.20 30.75 31.19	31.77 31.07 31.46
	0	31.54 30.81 31.54	41.49 31.31 31.89	32.29 31.73 31.74

Primary monocytes with IFN-β/IFN-γ

	IFNγ(U/ml)→ / IFNβ(U/mL)↓	100	10	1	0
Apo-E	100	31.37 30.78 31.65	30.88 330.45 30.59	30.80 30.24 31.09	30.91 30.62 30.19
	10	30.85 31.76 31.30	28.94 29.28 28.57	29.21 29.43 29.13	28.09 28.70 27.82
	1	29.96 29.40 30.23	28.36 27.96 28.10	28.98 28.44 29.25	27.90 27.64 27.26
	0	29.97 30.86 30.42	27.34 27.67 27.00	27.80 28.01 27.73	27.35 27.95 27.10
	IFNγ(U/ml)→ / IFNβ(U/mL)↓	100	10	1	0
G6PDH	100	27.78 28.23 28.62	28.42 28.73 28.60	28.20 28.06 28.71	29.08 28.53 28.24
	10	28.38 28.73 28.38	27.91 28.27 28.86	28.40 28.49 29.15	28.23 28.07 28.35
	1	29.05 29.97 29.32	28.57 28.48 28.04	28.35 28.14 28.63	27.94 28.78 28.10
	0	28.68 28.99 29.01	28.12 28.37 28.63	28.46 27.90 28.47	28.19 28.44 28.16