AN *IN VITRO* INVESTIGATION OF THE EFFECTS OF RIMONABANT (A CANNABINOID CB_1 RECEPTOR ANTAGONIST) ON CELL ADHESION AND INFLAMMATORY ASSOCIATED CYTOKINE PRODUCTION.

Adorée Bouwer

Student nr. 22203444

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The Faculty of Health Sciences

Department of Pharmacology

University of Pretoria

Pretoria

RSA

Abstract

There is good pharmacological evidence that cannabinoids caused cellular changes by interacting with specific cannabinoid receptors (CBR) (Klein *et al*., 2000). To date, two CBRs have been identified in the human body, designated Cannabinoid Receptor 1 (CB_1) and Cannabinoid Receptor 2 (CB_2) (Begg *et al*., 2005). Endogenously occurring compounds with action at the CBRs also exist and they are called endocannabinoids. One of the four known endocannabinoids is anandamide (AEA). The endocannabinoid system, present in the human body, plays a significant role in altering the physiology of the immune system. Enhancement of this system's anti-inflammatory effect could possibly present a vital therapeutic target for central and peripheral inflammatory disorders.

A number of synthetic CB_1 or CB_2 specific antagonists have been developed including the highly specific CB_1 receptor antagonist/reverse agonist named Rimonabant/ SR141716A. SR compounds are considered unique because these compounds not only inhibit the binding and function of cannabimimetic agents, but also act as inverse agonists. Activation of CB_1 receptors produces inappropriate CNS side effects including psychoactivity, dependence and sedation (Clayton *et al*., 2002) whereas CB_1 receptor antagonists/inverse agonists avoid or prevent these side effects. Taking the above information into consideration, Rimonabant has the potential to offer an effective long term treatment of chronic inflammatory disorders without the serious side effects of commonly used treatments.

The main aim of this study is to investigate the *in vitro* effects of Rimonabant alone and in combination with anandamide on inflammatory associated cytokine production by human umbilical vein endothelial cells (HUVEC) and macrophage cultures.

After careful consideration of the evidence stating that endothelial cells produce several important molecules vital to the inflammatory response of the body and the confirmation that CB_1 receptor mRNA is generally present in endothelial cells, the use of HUVEC was deemed to be satisfactory for this study. The first phase of the study was dedicated to

establishing the technique to isolate HUVEC from fresh human umbilical cord within the local laboratory and to maintain these in culture for further use during experimental procedures to test the effects of CB_1 ligands. The isolation procedure, trypsinising, freezing away and thawing methods used during this experiment produced healthy HUVEC in sufficient numbers for further use.

The next step was to determine the maximum *in vitro* concentrations at which Rimonabant and anandamide had insignificant cytotoxic effect on selected human cells and in doing so, determine suitable concentrations for further experimentation. Both compounds had a dose related anti-proliferative response when tested on HUVEC. The same dose related response was observed during the Rimonabant exposure to human lymphocytes, but no decrease in lymphocyte viability was observed when treated with anandamide at the concentrations tested. It is evident from the results that there was an almost ten times difference in the IC_{50} value of the two different products (14.3 μM for Rimonabant and 124.2 μM for anandamide) which was statistically significant.

Flow cytometry was used to determine the effects of Rimonabant and anandamide on the surface expression of the CR3 complement receptor by human neutrophils. Neither Rimonabant nor anandamide significantly affect CR3 expression on the surface of freshly isolated human neutrophils and would exclude the CR3 expression pathways as a potential mechanism of action for the anti-inflammatory effects of these compounds.

The *in vitro* effect of Rimonabant and anandamide alone and in combination on the production of cytokines by human macrophages and by HUVEC was determined. Anandamide was shown to inhibit the production of all the detectable cytokines (IL-8, IL-1β and IL-6 in both cell types and IL-10 and TNF-α in macrophages). Furthermore this inhibitory effect was attenuated by pre-treatment Rimonabant. These results would suggest that anandamide could induce anti-inflammatory effects observed in macrophages and HUVEC, through cannabinoid receptors. Rimonabant also inhibited the production of all the detectable cytokines following treatment with 0.5 μM and 3 μM respectively. The anti-inflammatory effects of anandamide were attenuated when

combined with 1 µM of Rimonabant. Throughout the various cytokine responses, the dose-response relationship appeared to follow a bell-shaped dose-response. This occurrence proposes that Rimonabant displaces anandamide and blocks the anti-inflammatory effects of the agonist.

Flow cytometry was used to determine the effects of Rimonabant and anandamide alone and in combination on the extracellular surface expression of ICAM-1 by HUVEC. Neither Rimonabant nor anandamide had any significant inhibitory effect on the expression of ICAM-1 by HUVEC. Considering the low levels for ICAM-1 expressed by the HUVEC during this experiment and the literature supporting more effective methods of activating the ICAM-1 gene and subsequent up-regulation of ICAM-1 proteins, TNF-α stimulation of HUVEC might produce a different result compared to IL-1β stimulation.

The final phase of the project was to determine the effects of Rimonabant and anandamide on the adhesion of human neutrophils to HUVEC. There was no significant difference with relation to the neutrophil adhesion to HUVEC following the treatment with various combination concentrations of the compounds, and also no significant effect following treatment with either test compound individually.

Although a specific mechanism of action for Rimonabant could not be uncovered during this study, there is evidence that several possible mechanisms can be excluded. The results support observations made by other researchers and the hypothesis that Rimonabant has anti-inflammatory effects. The results provide motivation for further experimentation to better understand these anti-inflammatory actions of Rimonabant.

Keywords:

Rimonabant, Acomplia, SR141716A, anandamide, cannabimimetic agents, cytokines, bell-shaped dose-response, inverse cannabimimetic effects.

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List of Abbreviations

A

AEA	Arachidonylethanolamide (Anandamide)
2-AG	2-arachidonoyl glycerol
AC	Rimonabant
AjA	Ajulemic acid

C

CB_1	Cannabinoid receptor 1
CB_2	Cannabinoid receptor 2
CBCr	Cannabichromene
CBD	Cannabidiol
CBN	Cannabinol
CBR	Cannabinoid receptor
cm	Centimetre
CNS	Central nervous system
CO_2	Carbon dioxide
COX	Cyclo-oxygenase
CR3	Compliment receptor 3

D

DMSO	Dimethylsulfoxide

E

ECGF	Endothelial cell growth factor
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked Immuno-Sorbant assay

F

FAAH	Fatty acid amide hydrolase
FCS	Foetal calf serum

G

g	Centrifugal force
GM-CSF	Granulocyte monocyte colony stimulated factor

H

HBSS	Hanks balanced salt solution
HU-210	[(-)-11-hydroxy-Δ^8 tetrahydrocannabinol-dimethylheptyl]
HUVEC	Human umbilical vein endothelial cells

I

IC_{50}	Inhibitory concentration that results in 50% cell growth inhibition
ICAM-1	Intracellular cell adhesion molecule-1
IFN	Interferon
IgG	Human immunoglobulin G
IL	Interleukin

L

LFA-1	Leukocyte functional antigen-1
LPS	Lipopolysaccharide

M

MAdCAM-1	Mucosal addressin cell adhesion molecule-1
µl	Microlitre
mM	Millimolar
mg	Milligram
µg	Microgram

µl/ml	Microlitre per millilitre
mg/ml	Milligram per millilitre
ml	Millilitre
µM	Micromolar
MAP	Mitogen-activated protein
MCP-1	Monocytes chemoattractant protein-1
MDP	Muramyl dipeptide
MPO	Myeloperoxidase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

N

NF-κB	Nuclear Factor κB
ng	Nanogram
ng/ml	Nanogram per millilitre
NK cells	Natural killer cells
NSAID	Non-steroidal anti-inflammatory drugs

P

PBS-	Phosphate buffered saline (without Ca^{++}, without Mg^{++}, without phenol red)
PBS+	Phosphate buffered saline (with Ca^{++}, with Mg^{++})
PG	Prostaglandin
pH	Negative log of the hydrogen ion concentration
PHA	Phytoheamagglutinine
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear leukocytes
P/S	Penicillin/Streptomycin cocktail for culture media

R

RPMI-1640	Rosswell Park Memorial Institute 1640 culture medium

S

SEM	Standard error of the mean
SR141716A	Rimonabant/Acomplia

T

THC	(-)-Δ^9-tetrahydrocannabinol
TNF-α	Tumour necrosis factor-α

V

VCAM	Vascular cell adhesion molecule

W

WIN 55212-2	{(R)-(+)-[2,3-dihydro-5-methyl-3- [(4-morpholinyl)methyl[pyrrolo[1,2,3-de]1,4- benzoxazin-6-yl] (1-naphthalenyl0 methanone}

Chapter 1: Introduction

1.1. Literature review

1.1.1. Inflammation

The immune system is a multifaceted, extremely adaptive defence system that provides protection against the invasion of pathogens, as well as, against injury (Teeling *et al*., 2008). The body's initial response to injury and trauma is manifested as inflammation, a function of innate immunity (Stites *et al*, 1991). Inflammation is traditionally defined by four symptoms: heat, pain, redness and swelling, all of which reflect the effects of cytokines and other inflammatory mediators on the local blood vessels (Janeway *et al*, 2005). An inflammatory response can be generated from cells (neutrophils, eosinophils, basophils, macrophages, mast cells, endothelium and platelets) and from circulating proteins (components of the complement system, coagulation, fibrinolysis and kinin pathways) (Stites *et al*, 1991). The body has a cellular inflammatory response to defend itself against infection (pathogenic organisms such as viruses and bacteria) and to repair tissue damage (Figure 1.1). This defence response however, can result in a chronic disease status if inflammation persists. The cellular inflammatory response is rapidly activated in the body by inflammatory mediators, which are normally stored in cytoplasmic granules or to be newly generated from phospholipids available in the cell membrane. There are both a circulating and a non-circulating cellular inflammatory response and thus a distinction can be made between short-lived circulating inflammatory cells (neutrophils, eosinophils, basophils) and cells that pre-exist in the tissue as long-lived resident non-circulating inflammatory cells (mast cells and macrophages) (Stites *et al*, 1991).

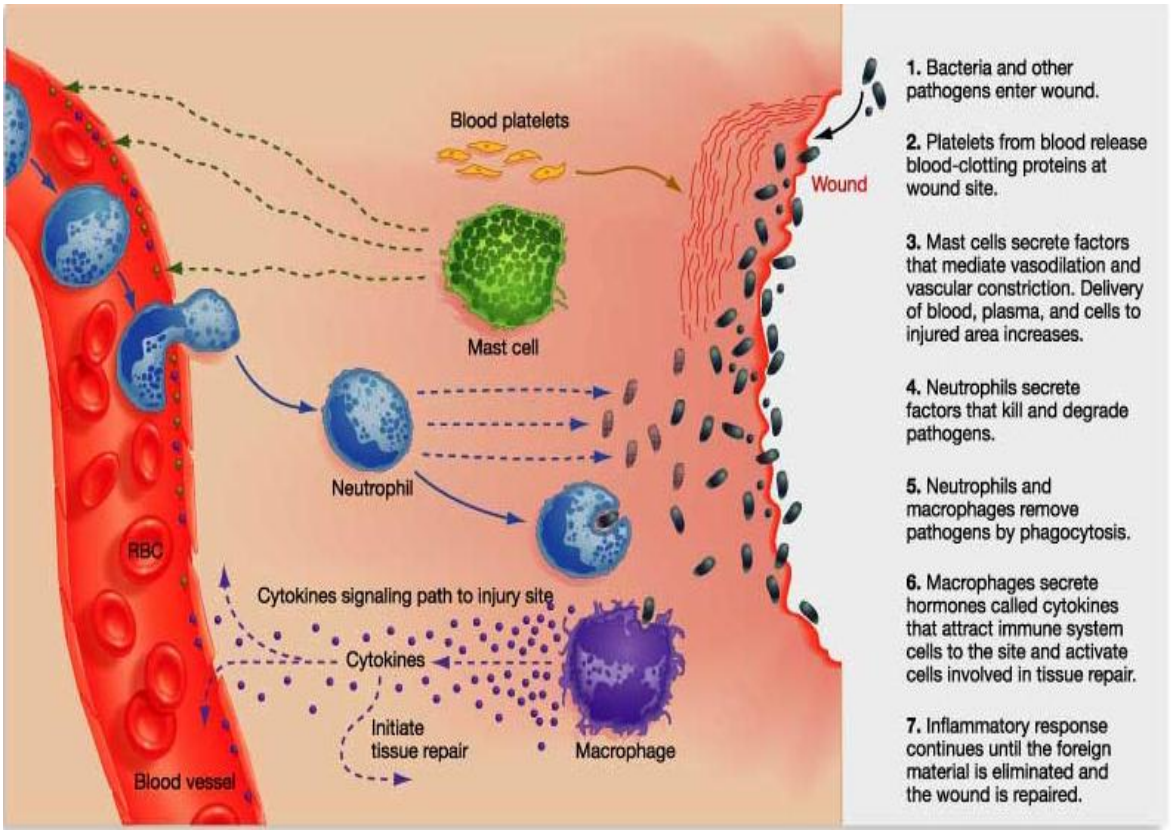


Figure 1.1. A diagram representing the body's response to injury (Muller, 1998).

The different inflammatory cells have different primary functions e.g. neutrophils and macrophages are phagocytes (their primary function is phagocytosis) whereas mast cells and basophils secrete inflammatory mediators. Phagocytes eliminate particles or organisms that have gained access to the host and act as a protective barrier between the environment and the host (Figure 1.2). The secretory cells (mast cells and basophils) on the other hand, contain both inflammatory mediators and chemotactic factors, which recruit inflammatory cells. The secretory cells thus contribute to the host's defence either by amplifying the effects of the phagocytic cells or by having a direct effect on target cells (Stites *et al*, 1991).

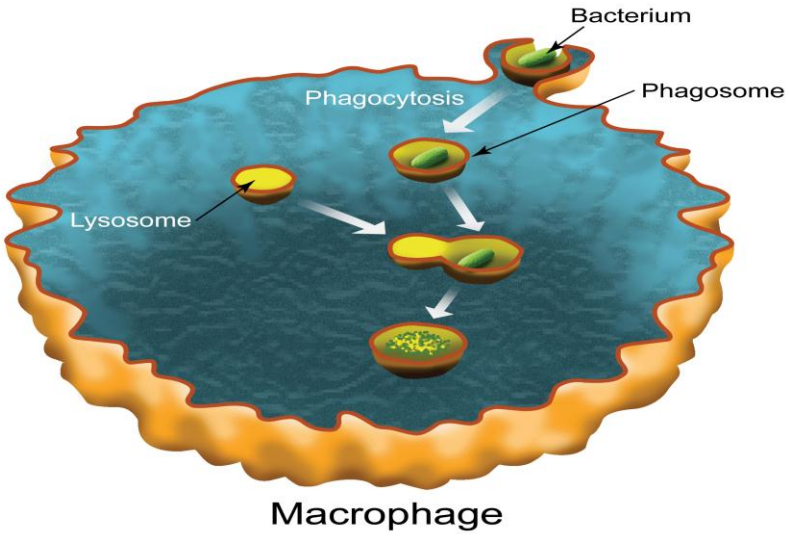


Figure 1.2. A diagrammatic representation of phagocytosis of an invading bacterium (www.newscentre.lbl.gov).

Nuclear factor (NF) κB is a ubiquitous transcription factor that plays a key role in the transcription of cellular genes regulating the inflammatory response of the body. NF-κB increases the expression of several genes that encode many pro-inflammatory cytokines, chemokines, enzymes that generate mediators of inflammation, immune receptors and adhesion molecules involved in chronic inflammatory diseases (Schottelius *et al*., 1999). NF-κB in located in the cytoplasm of unstimulated cells, bound to IκBα and IκBβ. This pair of proteins prevents NF-κB from entering the nuclei of the cells. When these cells are stimulated, specific kinases phosphorylate IκB causing its rapid degradation by proteasomes. This process results in the release of NF-κB into the nucleus of the cell where it binds to target genes, thereby activating gene expression (Barnes *et al*., 1997). The activation of NF-κB therefore mediates inflammatory and immune responses.

1.1.2. Cannabinoid receptors

In the late 1980s, there was good pharmacological evidence that cannabinoids caused cellular changes by interacting with specific cannabinoid receptors (CBR) (Figure 1.3) (Klein *et al*., 2000). A receptor is defined as a macromolecule to which another macromolecule/drug binds to elicit a specific physiological response (Sommers, 2002).

The binding of the cannabinoids to their specific receptors allows these cannabinoids to induce defined biological effects.

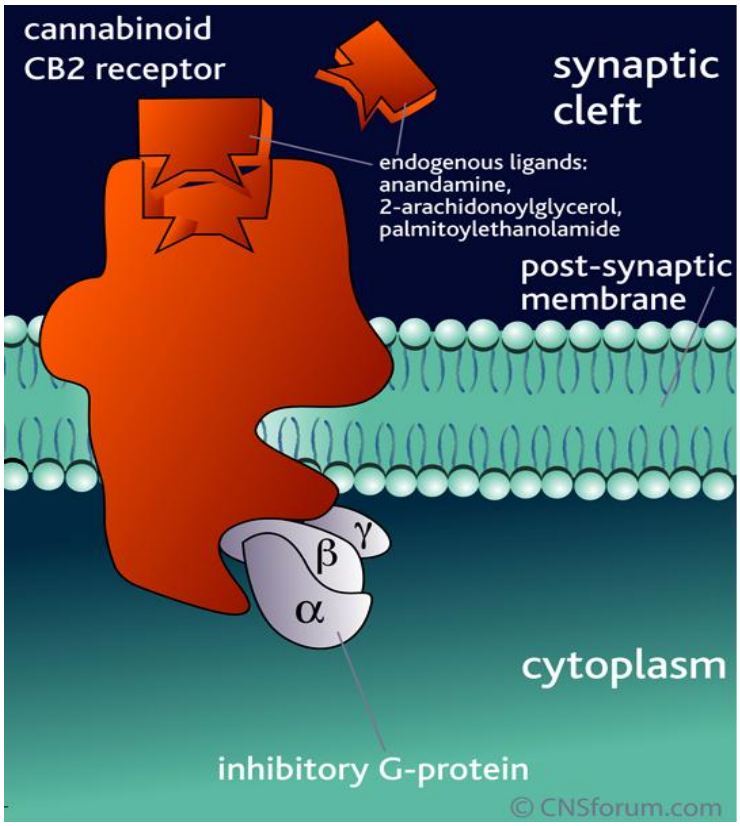


Figure 1.3. A diagrammatic representation of cannabinoids binding to cannabinoid receptor 2 (CB_2) on a neural cell membrane (www.cnsforum.com).

To date, two CBRs have been identified in the human body by molecular cloning, designated Cannabinoid Receptor 1 (CB_1) and Cannabinoid Receptor 2 (CB_2) (Figure 1.3) (Begg *et al*., 2005). The CB_2 receptor shares only 44% homology with CB_1. Unlike CB_2, CB_1 is primarily expressed in the brain hippocampal formation, basal ganglia and the molecular layer of the cerebellum, probably accounting for the psychoactive effects of cannabinoids. CB_1 receptors have also been detected in testis, spleen cells and leukocytes, with lower levels found in B lymphocytes, natural killer cells (NK cells), polymorphonuclear leukocytes (PMNs), monocytes and $CD4^+$ cells. However, the function of these cannabinoid receptors expressed in these tissues is unclear. CB_2

receptors, on the other hand, are found mainly on immune cells such as B lymphocytes (bone marrow programmed lymphocytes), T lymphocytes (thymus programmed lymphocytes), macrophages and mast cells, thus indicating that they might play a role in the modulation of cytokine release (Sacerdote *et al*., 2000). The expression of CB_2 receptors in immune cells suggests that the majority of the immunomodulatory properties of cannabinoids might be mediated through CB_2 receptors; however, this theory is supported by very few studies (Ihenetu *et al*., 2003). In a publication by Calignano *et al*. (1998) it was concluded that CB_1 receptors are implicated in nociceptive pain whereas CB_1 and CB_2 receptors are implicated in inflammatory hypersensitivity.

Both CB_1 and CB_2 receptors are members of the heptahelical G protein-coupled receptor superfamily (Figure 1.3). The CB_2 receptor is known to mediate its effects mostly through the pertussin toxin-sensitive $G_{i/o}$ inhibition of adenylyl cyclase, although it can also mediate βγ-mediated intermediate-early gene expression through the mitogen-activated protein (MAP) kinase pathway. The CB_1 receptor inhibits N- and Q- type voltage-dependent Ca^{2+} channels and stimulates inwardly rectifying K^+ currents in addition to having the same functions as the CB_2 receptor (Calandra *et al*., 1999).

An understanding of the differences and distribution of the CB receptors in the human body provides a guideline as to which of the two receptors are probably involved in the anti-inflammatory actions caused by the specific binding of the cannabinoids to these receptors.

1.1.3. *Cannabis sativa*

Cannabis sativa, popularly known as Cannabis, is an annual plant in the Cannabaceae family. *Cannabis sativa* has been the source of medicinal preparations for many years and has been a topic of interest in pharmacobotany since the earliest written records. *Cannabis sativa* is considered a complex botanical specimen due to its combination of more than 60 cannabinoids, as well as, the 200-250 non-cannabinoid constituents. Several studies done over many years demonstrate this plant's therapeutic benefits, and focus was drawn to the potent anti-inflammatory actions of a crude marijuana extract

containing ((-)-Δ^9-tetrahydrocannabinol (THC) (Zurier, 2003). Cannabinoids have many medicinal uses including pain relief in paraplegia, neuralgia and spasticity in multiple sclerosis, the prevention of nausea and vomiting associated with cancer chemotherapy, immunomodulators for auto-immune diseases and alleviates allergies and nephritis (Onaivi *et al*., 1999). The immunomodulatory properties of cannabinoids are well established and numerous reports propose that cannabinoids demonstrate immunomodulatory properties due to their effect on a variety of inflammatory cells (Ihenetu *et al*., 2003). The broad array of effects that cannabinoids have on the immune system includes inhibition of the innate, humoral and cell-mediated immune responses (Kaminski, 1998).

1.1.4. ((-)-Δ^9-tetrahydrocannabinol (THC)

According to Spoto *et al*. (2006), THC is the active compound of *Cannabis sativa* preparations like hashish and marijuana. THC was the first cannabinoid ligand to be structurally defined and synthesized (Klein *et al*., 2000). Studies done on rodent cells show that Δ^9-THC modulates the production of a number of cytokines including interferons (IFNs), TNF-α, IL-1 and IL-2. However, it has been proven that Δ^9-THC not only effects cytokine production but also inhibits the proliferative responses of T lymphocytes, antibody synthesis, antimicrobial activity of macrophages, cytotoxic T-cell activity, NK cell toxicity for target tumour cells, as well as down regulates the production of IFN-γ (Patrini *et al*., 1997). More recent studies done on a wider selection of cannabimimetic agents ranging from marijuana to cannabinoid analogues such as HU-211, displayed a much larger diversity of cytokines affected by the drugs. These studies showed that cannabimimetic agents not only have an effect on interleukins but also on chemokines (Klein *et al*., 2000). Klein *et al*. (2000) also demonstrated pro-inflammatory and anti-inflammatory effects of THC on cell subpopulations ranging from T lymphocytes to eosinophils. That study measured the production of cytokines and chemokines by these cell populations in the presence of THC. Anti-inflammatory effects were observed in the various cell types with TNF-α, granulocyte monocyte colony stimulated factor (GM-CSF) and IFNγ all decreasing due to drug treatment. However, pro-inflammatory effects were also observed in that chemokines, especially IL-8 were

increased and the anti-inflammatory cytokine, IL-10, was decreased following THC treatment. That study thus concluded that different immune cell subpopulations have varying thresholds of responsiveness to THC and other cannabimimetic agents, some of which are probably CBR mediated and some that are not (Klein *et al*., 2000).

Since THC is the psychoactive agent of *Cannabis sativa*, many physicians are reluctant to prescribe pure THC for fear of substance abuse in vulnerable populations. However, further studies done on rats indicated that cannabidiol (CBD), cannabichromene (CBCr) and cannabinol (CBN), all of which are non-psychoactive *Cannabis sativa* constituents, proved to have an anti-inflammatory effect on acute inflammation (Figure 1.4). More recently, CBD was shown to reduce joint inflammation in a murine model of collagen-induced arthritis (Zurier, 2003).

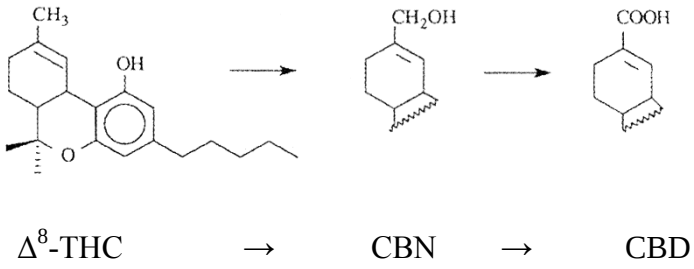


Figure 1.4. The major pathway of metabolism for Δ^9-THC, cannabinol and cannabidiol (Burstein, 1999).

The discovery of carboxy THCs, a class of cannabinoids free of cannabimimetic central nervous system activity, produced promising results and attracted scientists' attention worldwide. These cannabinoid acids, which are metabolites of THC, do not produce behavioural changes in humans even when given at doses several times greater than THC, making them promising therapeutic agents. The term cannabinoid acid includes all the carboxylic acid metabolites of the cannabinoids and their synthetic analogues (Burstein, 1999). According to Burstein (1999), THC is rapidly metabolised in the body to a number of oxygenated acid metabolites. Due to the prevalence and persistence of these acid metabolites in human tissue, they have become the basis for most of the drug abuse screening methods for the detection of Cannabis usage. Thus, regular Cannabis

users are chronically exposed to moderately high plasma levels (65-70 ng/ml) of Δ^9-THC-11-oic acid. Burstein (1999) also reported that direct administration of Δ^9-THC-11-oic acid to human subjects produced no psychotropic responses and resulted in very little further metabolism of the acid other than the formation of glucuronide conjugates.

Zurier (2003) stated that THC-11-oic acid is effective in animal models of inflammation and pain at oral doses of 20-40 mg/kg and it was shown to suppress both the cyclooxygenase and lipoxygenase activities of cells in tissue culture. Furthermore, Burstein (1999) reported that Δ^9-THC-11-oic acid inhibits one of the *in vitro* effects of Δ^9-THC. Δ^9-THC-11-oic acid was able to reduce Δ^9-THC-induced synthesis of immunoreactive prostaglandin PGE_1 in WI-38 human lung fibroblasts, most likely by inhibiting the action of cyclooxygenase (COX). Inhibition of PG synthesis is a typical property of non-steroidal anti-inflammatory drugs (NSAIDs) and this observation suggested that Δ^9-THC-11-oic acid might exhibit similar effects in experimental models of pain and inflammation (Burstein, 1999). THC-11-oic acid, however, lacks sufficient activity to be considered for clinical use (Zurier, 2003).

1.1.5. Endocannabinoids

Scientists' interest in cannabinoid research intensified in the early 2000's due to the cloning of specific cannabinoid receptor subtypes and the discovery of endogenous ligands (Calandra *et al.*, 1999). Endogenously occurring compounds with action at the CBRs are called endocannabinoids. According to Spoto *et al.* (2006), endocannabinoids are agonists of CB_1 or CB_2 cannabinoid receptors and as such they are able to mimic several effects of Δ^9-THC. Endocannabinoids are amides, esters and ethers of long chain polyunsaturated fatty acids and the discovery of four endocannabinoids, which may modulate neural, vascular and immune cell function, reinforces the need for research into the regulatory activities of these endocannabinoids and similar molecules (Spoto *et al.*, 2006).

The four known endocannabinoids include 2-arachidonoyl glycerol (2-AG), 2-arachidonyl glyceryl ether, o-arachidonoyl ethanolamine and arachidonylethanolamide

(AEA) of which AEA and 2-AG are the most-studied members of this class of bioactive lipids (Zurier, 2003). Arachidonylethanolamide is also known as anandamide or AEA (Figure 1.5).

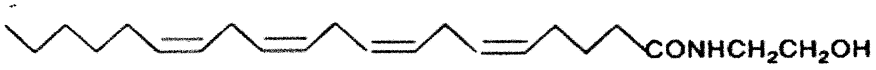


Figure 1.5. A structural representation of anandamide (AEA) (Burstein, 1999).

AEA is not considered a cannabinoid but rather an eicosanoid. AEA is produced in the brain and select peripheral tissues such as the spleen. It is available in the areas where receptors are present thus facilitating a role in endogenous cannabimimetic activity in the various areas of the body. AEA is also produced by cells of the immune system such as macrophages and other leukocytes (Spoto *et al*., 2006). AEA shares many, but not all of the *in vivo* effects of Δ^9-THC. One major difference between AEA and exogenous cannabinoids is that AEA has a relatively short duration of action when compared to long-acting plant derived cannabinoids. The termination of AEA action could be due to the fact that AEA is rapidly metabolised to inactive metabolites. This metabolic inactivation occurs through hydrolysis and is mediated by the action of fatty acid amide hydrolases (Wiley *et al*., 2006).

Studies indicate that AEA inhibits sensory neurotransmitter release both *in vivo* and *in vitro* (Clayton *et al*., 2002). According to a study done by Clayton *et al*. (2002), AEA (a CB_1 receptor agonist) inhibits the early phase of the formalin pain response involving C-fibre activation. However, the stable analogue of AEA, methanandamide, inhibited both the early and late phases of the response associated with inflammation and central sensitisation. DiMarzo *et al.* (2002) noted that the endocannabinoids interact with non-CB_1, non-CB_2 G protein coupled receptors and several ion channels. A study done by De Petrocellis *et al*. (1998), demonstrated that AEA (as well as other CBR ligands) inhibited the proliferation of the human breast cancer cell lines (i.e. MCF-7 and EFM-19) through a mechanism involving prolactin. The publication concluded that the anti-proliferative effect of AEA was not due to enhanced apoptosis but rather a reduction in the number of

cells entering the S phase of the cell cycle, which indicated that the anti-proliferative effect was CB_1 receptor mediated and the mitogenic effect was inhibited by AEA (De Petrocellis *et al*., 1998).

AEA treatment has also been reported to increase cytokine-induced proliferation as seen in a publication by Valk *et al*. (1999). In that study they cultured normal mouse bone marrow cells in the presence of IL-3 and AEA and found that more hematopoietic colonies were produced when compared to culturing with IL-3 only. Furthermore, the myeloid cell line, 32Dc13, proliferated to a greater extent in the presence of AEA and this "growth factor effect" was observed when AEA was co-cultured with factors other than IL-3, such as GM-CSF, G-CSF and erythropoietin. Ironically, this AEA effect is not shared by other cannabimimetic agents, and the effect appeared to be dependent upon the expression of CB_2 receptors (Valk *et al*., 1999). CBR-independent growth-promoting effects of AEA in hematopoietic cell cultures (in the presence of either IL-6 or IL-3) have also been reported by other investigators. AEA was also shown to stimulate MAP kinase activity and similar to proliferation this effect was not blocked by the synthetic cannabinoid compounds (as described in Section 1.1.6 below). From these results, the authors concluded that AEA was activating biological processes in a non cell-surface receptor-mediated way, which is not entirely unexpected for long chain fatty acid compounds that can readily traverse the cell membrane. It is thus apparent that endogenous cannabimimetics participate in the growth factor-induced maturation and differentiation of hematopoietic cells and in so doing can have an effect on the growth and development of the immune function (Klein *et al*., 2000).

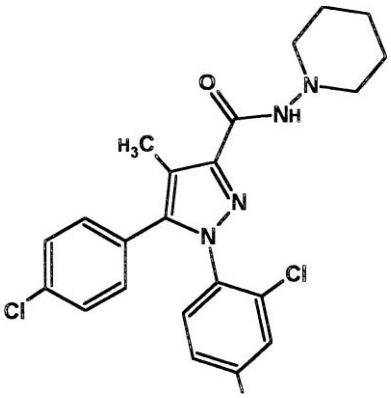
The endocannabinoid system, present in the human body, plays a significant role in altering the physiology of the immune system. Enhancement of this system's anti-inflammatory effect could possibly present a vital therapeutic target for central and peripheral inflammatory disorders.

1.1.6. Synthetic cannabinoid receptor compounds (SR)

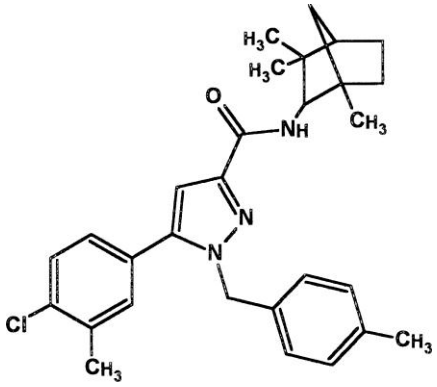
Several compounds, including agonists (a pharmacologically active drug at a specific site/receptor) and antagonists (a drug which either diminishes the effect of an agonist or abolishes it completely) for the CBRs, have recently been synthesized for research purposes and are being used widely to gain more knowledge about the distribution and function of CBRs. Compounds possessing agonist activity at CB_1 receptors include; WIN55212-2, CP55,940 and HU-210 and it has been found that they each inhibit intra-cellular adenylyl cyclase activity with varying potencies (Egerton *et al*., 2006).

A number of synthetic CB_1 or CB_2 specific antagonists have been developed including the highly specific CB_1 receptor antagonist/reverse agonist named Rimonabant, and originally known as SR141716A having the chemical structure *N*-(piperidin-1-yl)-5-(4-chloro-phenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrozole-3-carboxamide hydro-chloride and commercially available as "Acomplia" (Figure 1.6). The selective CB_2 antagonist SR144528 has the chemical structure of N-[(1S)-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carbox-amide (Calandra *et al*., 1999) is also shown in Figure 1.6.

SR compounds are considered unique because these compounds not only inhibit the binding and function of cannabimimetic agents, but also act as inverse agonists. Thus, SR compounds alone may cause changes in biological function by suppressing the constitutive activity of CBRs, as well as the activity of other G-protein coupled receptors (Klein *et al*., 2000).



SR141716A (Rimonabant)



SR144528

Figure 1.6. The chemical structures of SR141716A (specific to CB_1) and SR144528 (specific to CB_2) (Klein *et al*. 2000).

1.1.7. Inverse cannabimimetic effects

Studies indicate that some CB_1 or CB_2 receptor-containing systems also produce "inverse cannabimimetic effects". These "inverse cannabimimetic effects" are effects opposite in effect from those produced by cannabinoid receptor agonists (Pertwee. 2005). SR141716A (Acomplia/Rimonabant), an orally active compound, has a much higher affinity for CB_1 receptors when compared to its affinity for CB_2 receptors. According to Klein *et al*. (2000), a test done to determine the ability of Rimonabant to inhibit the effect of an agonist using rat brain (CB_1 rich) and splenocyte membrane (CB_2 rich) preparations that were treated with an appropriate agonist and Rimonabant, resulted in a much greater inhibition of the agonist in the CB_1 rich tissue (Klein *et al*., 2000). In addition, when administered orally to mice in low doses, Rimonabant inhibited a series of agonist-induced effects including anti-nociception (Klein *et al*., 2000). Rimonabant can cause responses in tissues that are opposite in direction from those produced by CB_1 receptor agonists and can thus be described as a CB_1 antagonist/inverse agonist (Pertwee *et al*., 2002). However, there is also evidence that it can induce inverse cannabimimetic effects at sites without CB_1 receptors (Pertwee, 2005). Furthermore, it is likely that any inverse cannabimimetic effects are produced through receptor-independent mechanisms that require higher concentrations than those necessary to merely displace endocannabinoids from their receptors (Pertwee, 2005).

1.1.8. Previous anti-inflammatory studies involving Rimonabant

According to Pertwee *et al*. (2002), *in vivo* and *in vitro* studies indicated that Rimonabant was more potent in blocking the action of CB_1 receptor agonists than causing an inverse cannabimimetic response. This finding may be because Rimonabant binds with higher affinity to the agonists binding site than it does to the site responsible for the inverse cannabimimetic response.

Croci *et al.* (2004) described a role for cannabinoid CB_1 receptors in the gut of rodents and found the CB_1 antagonist, Rimonabant, promotes anti-inflammatory activity. Inflammation was induced in the small intestines of rodents with either indomethacin or lipopolysaccharide (LPS). In the case of indomethacin-induced ulcers, Rimonabant reduced the ulcers in rats as well as increasing TNFα release and myeloperoxidase (MPO) activity. A wild type mouse and a CB_1 receptor knockout mouse strain was used during this experiment and ulcers induced by both indomethacin and LPS produced the same results. Although Rimonabant reduced intestinal lesions in both models, no effect was observed for the TNFα levels produced in either of the mouse models used.

In a study done by Patrini *et al*. (1997) on male Sprague Dawley rats, Rimonabant significantly suppressed the immunological functions of the rats and compared well to a cannabinoid agonist (CP-55,940) in inhibiting the immune parameters. This study suggested the involvement of CB_2 receptors rather than CB_1 receptors, which implies that the Rimonabant might be of therapeutic value in chronic inflammatory diseases.

Using the knowledge and methods of receptor targeting drug design, it is now possible to separate the psychoactivity (caused by THC) and medicinal properties of *Cannabis sativa*. Activation of CB_1 receptors produces inappropriate CNS side effects including psychoactivity, dependence and sedation (Clayton *et al*., 2002) whereas CB_1 receptor antagonists/inverse agonists avoid or prevent these side effects. Taking the above information into consideration, Rimonabant has the potential to offer an effective long term treatment of chronic inflammatory disorders without the serious side effects of commonly used treatments.

1.2. General outline of this study

1.2.1. Hypothesis

Rimonabant, a CB_1 antagonist, has measurable *in vitro* anti-inflammatory properties when tested on human umbilical vein endothelial cell (HUVEC) or lymphocyte cultures.

1.2.2. Aim of the study

The main aim of this study is to investigate the *in vitro* effects of Rimonabant alone and in combination with anandamide on inflammatory associated cytokine production by HUVEC and macrophage cultures.

A secondary aim is to investigate the effect of Rimonabant on neutrophil adhesion *in vitro*.

1.2.3. Study objectives

Stage one

The objectives of this stage of the study were:

1. To establish a method to isolate and culture primary human umbilical vein endothelial cells (HUVEC) in the Department of Pharmacology, University of Pretoria.
2. To determine the effects of Rimonabant and anandamide on the proliferation of HUVEC.
3. To determine the effects of Rimonabant in combination with anandamide on the proliferation of human lymphocytes.

Stage two

The objectives of this stage of the study were:

4. To determine the effects of Rimonabant and anandamide (at non-toxic concentrations) on the expression of the CR3 adhesion molecule by human neutrophils using a flow cytometric method.

5. To determine the effects of Rimonabant and anandamide, at non-toxic concentrations, on the production of pro-inflammatory cytokines by resting and stimulated HUVEC and isolated macrophages using a flow cytometric bead assay.
6. To determine the effects of Rimonabant and anandamide, at non-toxic concentrations, on ICAM-1 expression by HUVEC.
7. To determine the effects of Rimonabant and anandamide, at non-toxic concentration, on the adhesion of human neutrophils to stimulated HUVEC.

Chapter 2: Isolation and culturing of Human Umbilical Vein Endothelial Cells (HUVEC)

2.1. Introduction

2.1.1. The umbilical cord

The human umbilical cord is the connection between the placenta and the embryo and is formed by the fifth week of gestation. The umbilical cord protects the blood vessels that travel between the embryo/foetus and the placenta throughout the duration of the pregnancy. A normal umbilical cord contains two umbilical arteries and one umbilical vein embedded in a loose, proteoglycan rich matrix known as Wharton's jelly (Figure 2.1).

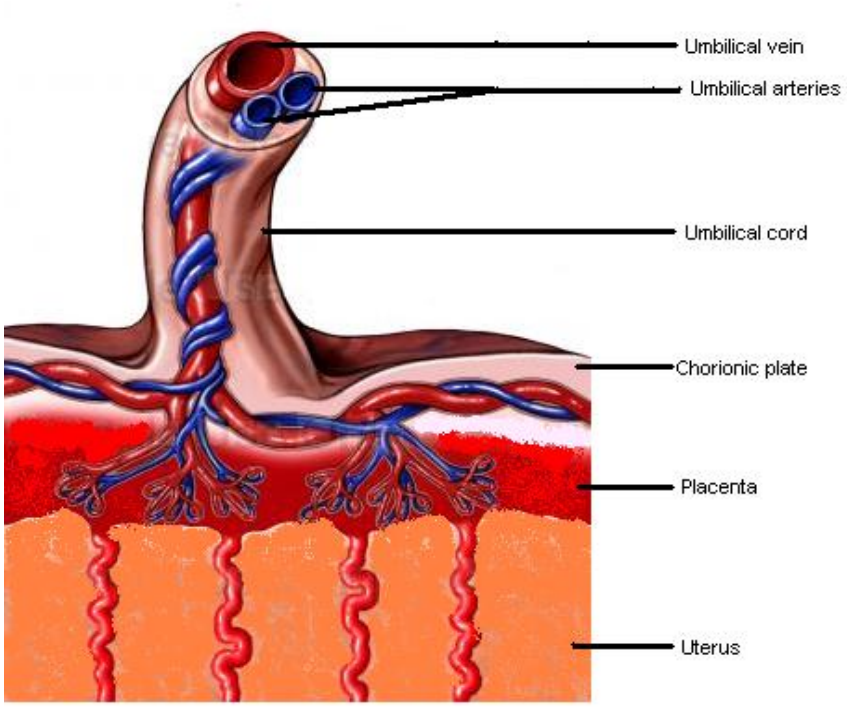


Figure 2.1. A structural representation of the human umbilical cord connected to the placenta (http://findlaw.doereport.com/generateexhibit.php).

The Wharton's jelly has physical properties that are resistant to twisting and compression and thus protects the essential vascular lifeline between the placenta and the embryo/foetus (Kliman, 2005). The blood vessels of the umbilical cord are lined with endothelium containing intercellular spaces (which interdigitate with one another), as well as pinocytotic vesicles (Hoang-Ngoc *et al.*, 1985).

The umbilical arteries can function not only as tubes for conducting blood, but also as organs that regulate the blood flow. This is possible due to their thick muscular tunic, many elastic fibres and the ability of their lumen to dilate (Figure 2.2). The umbilical vein on the other hand, has a thin muscular wall unable to stretch and thus behaves as an organ to return the blood flow (Figure 2.2) (Hoang-Ngoc *et al.*, 1985).

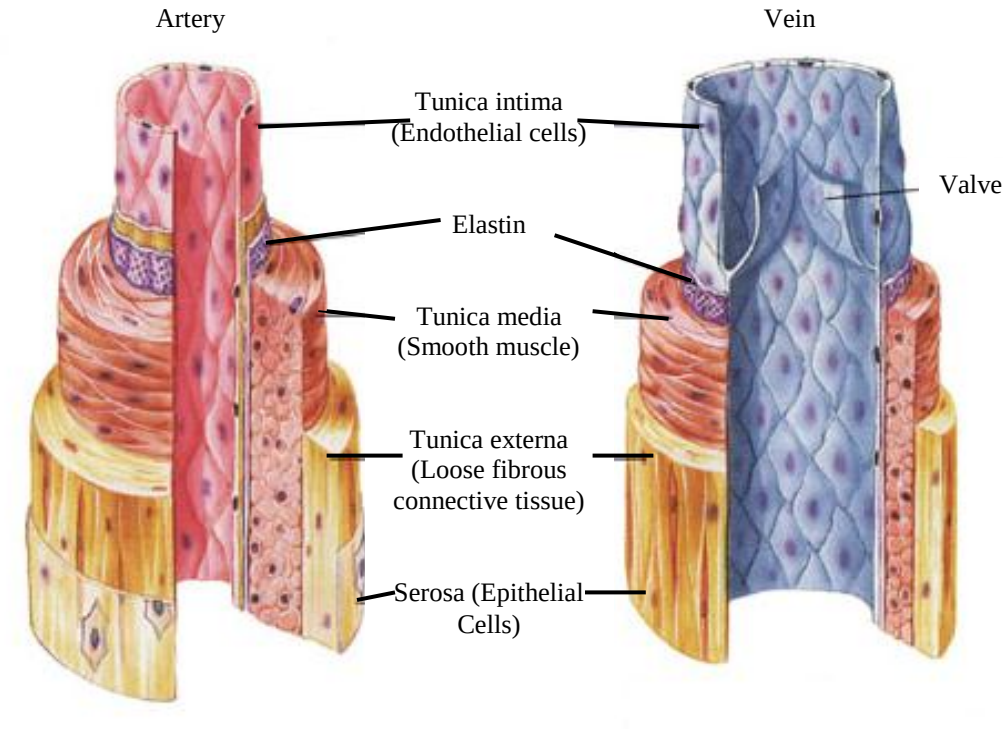


Figure 2.2. The difference between the structure of a human artery and a vein (Fox, 1993).

2.1.2. Endothelial cells

Endothelial cells line all blood and lymphatic vessels in the body. Endothelial cells are flat polygonal cells, which are connected to each other by junctional complexes.

Endothelial cells have many metabolic functions including the production of molecules, which mediate an acute inflammatory reaction (Young *et al*., 2000). Brown *et al*. (1994) proved that endothelial cells contribute actively to local vascular inflammatory responses. The molecules produced by endothelial cells, include IL-1, IL-6, IL-8 and cell adhesion molecules (Young *et al*., 2000).

The activation of the vascular endothelium is a critical early event in the development of inflammation and if persistent can lead to atherosclerosis. The activation of the endothelium initiates several actions that lead to inflammation including the increased adhesion of circulating monocytes to the injured endothelial layer, the infiltration of the monocytes into the vessel wall and the differentiation of the monocytes into macrophages (Kim *et al*., 2006). CB_1 receptor mRNA has been detected in endothelial cells from the human aorta, hepatic artery and in an endothelial cell line derived from umbilical vein endothelial cells (Liu *et al.*, 2000).

Endothelial cells play a vital role in the body's inflammatory response. According to Sadeghi *et al.* (2000), endothelial cells express adhesion molecules such as ICAM-1 to facilitate the adhesion of cells, such as neutrophils, to allow for migration towards damaged tissue and are known to produce and release certain cytokines. The endothelial cells are activated by exposure to pro-inflammatory cytokines such as IL-1 and TNFα (Sadeghi *et al.*, 2000).

A wide variety of endothelial cells from different organs including glioblastoma endothelial cells (Schley *et al*., 2009) and murine brain endothelial cells (Mestre *et al*., 2006) have been used during experiments to improve the knowledge of the effects and method of action of various cannabinoid drugs due to the high level of CB_1 and CB_2 receptors present on different endothelial cells.

After careful consideration of the evidence stating that endothelial cells produce several important molecules vital to the inflammatory response of the body and the confirmation that CB_1 receptor mRNA is generally present in endothelial cells, the use of this cell type

was deemed to be satisfactory for this study. Primary HUVEC in culture were selected as the cell type to be used in the experiments intended to determine the possible anti-inflammatory properties of Rimonabant.

2.2. Aim

The aim of this phase of the study was to establish the technique to isolate human umbilical vein endothelial cells from fresh human umbilical cord within the local laboratory and to maintain these in culture for further use during experimental procedures to test the effects of CB_1 ligands.

2.3. Materials and methods

The donor patients used in this study were carefully selected according to predetermined inclusion and exclusion criteria to ensure disease free HUVEC for the experiments. Ethical approval of this study was obtained from the Research Ethics Committee of the Health Sciences Faculty of the University of Pretoria under protocol number S36/2006. The inclusion and exclusion criteria for the patients selected for umbilical cord collection are as follows:

Inclusion criteria:

- Patients over the age of 18.
- Patients undergoing a caesarean section.

Exclusion criteria:

- Patients that are HIV positive.
- Patients suffering from any illness or disease.

Each patient was required to sign an informed consent form prior to the caesarean section verifying their authorization for the use of their umbilical cord during this research project (refer to Annexure A).

2.3.1. Preparation of reagents

All the reagents were sterilised before use and contamination was avoided by ensuring that the reagents were sealed properly for storage and only opened inside a sterilised laminar flow cabinet (Labotec, Model LCA 6000VA).

i. Saline+

NaCl (0.9 g) was dissolved in distilled water (1000 ml). This solution was sterilized by autoclaving it for 30 minutes at 121^{o}C. Ten millilitres of penicillin/streptomycin solution (cat no. 17-602E) supplied by The Scientific Group (Johannesburg, RSA) was added to the sterile solution. In addition 0.1 g of ethylenediamine tetra-acetate (EDTA), from Sigma Aldrich (St Louis, USA), was added to the sterile solution.

ii. Hanks Balanced Salt Solution (HBSS)

Sterile Hanks Balanced Salt Solution (without Ca^{++}, without Mg^{++}, without phenol red) was purchased from Highveld Biological. Five millilitres of penicillin/streptomycin solution, supplied by The Scientific Group (Johannesburg, RSA) was added to 500 ml HBSS before use.

iii. Collagenase

Collagenase type I from *Clostridium histolyticum*, purchased from Sigma Aldrich (St Louis, USA) was dissolved in HBSS (without Ca^{++}, without Mg^{++}, without phenol red) at 1 mg/ml. This solution was filter sterilized (0.2 µm pore size) syringe filter and stored at 4^{o}C in the dark (collagenase is light sensitive).

iv. Calcium and magnesium – free buffered saline solution (PBS-)

A ten times concentrate of PBS was made, sterilised and stored. This was diluted 1/10 just before use. Sigma Aldrich (St Louis, USA) supplied the necessary reagents including NaCl, KCl, Na_2HPO_4, KH_2PO_4 and Dextrose. These compounds were weighed out and dissolved in 900 ml deionised water.

NaCl	80.0 g
KCl	2.0 g
Na_2HPO_4	14.4 g
KH_2PO_4	2.4 g
Dextrose	2.0 g

All the salts were dissolved in 900 ml deionised water and pH adjusted to 7.4 and made up to 1 L before being filtered through Whatman filter paper. The solution was then bottled in 50-100 ml volumes and each filter sterilised (0.2 µm pores) followed by storage at room temperature as concentrated stock. Before use, 100 ml concentrated stock was diluted in 900 ml sterile deionised water and 10 ml of penicillin/streptomycin solution supplied by The Scientific Group (Johannesburg, RSA) was added to 1000 ml of PBS.

v. Bovine foetal calf serum (FCS)

FCS manufactured by Delta Bio-products, cat no. 14-501BI was supplied by The Scientific Group (Johannesburg, RSA). FCS was heat inactivated for 45 minutes at 56^{o}C.

vi. 199 Medium+

Sterile 199 culture medium was supplied by Sigma Aldrich (St Louis, USA). The medium was stored at 4^{o}C until use. Just before use the medium was supplemented with 20% FCS, 2 mM of L-Glutamine (supplied by Merck, Darmstadt, Germany) and 5 ml of penicillin/streptomycin solution supplied by The Scientific Group (Johannesburg, RSA).

vii. Heparin

Heparin was obtained from Sigma Aldrich (St Louis, USA). Heparin was dissolved at 3 mg/ml in distilled water and sterilised through 0.2 µm pore size syringe filter. The sterile heparin solution was stored in sterile bottles and stored at 4^{o}C.

viii. Endothelial cell growth factor (ECGF)

Sterile ECGF was obtained from Sigma Aldrich (St Louis, USA) and used undiluted.

ix. Gelatine

Gelatine from bovine skin, Type B was obtained from Sigma Aldrich (St Louis, USA) and used as a 1% solution. Distilled water was heated gently and stirred with a magnetic stirrer to allow the gelatine to dissolve completely. Once the gelatine dissolved completely the mixture was removed from the heat. The mixture was filter sterilised and stored at 4^{o}C until use.

x. Dimethylsulfoxide (DMSO)

Sterile DMSO manufactured by Acros Organics (New Jersey USA), was supplied by Sigma (Johannesburg, RSA) and used undiluted.

xi. Freeze media

Thirty seven and a half millilitres of 199 medium+ was mixed with 10 ml FCS and 2.5 ml DMSO

xii. Trypsin

Sterile TrypEL™ Express stable trypsin replacement, supplied by Gibco® was used undiluted.

2.3.2. Experimental procedures

The procedures used to isolate HUVEC are similar to the procedures described by Jaffe *et al*. (1973).

i. Procedures in theatre

Following the approval of the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria no. S36/2006 (refer Annexure B), as well as the approval of the CEO of Pretoria Academic Hospital and the Head of the Department of Obstetrics and Gynaecology, umbilical cords were collected from the Pretoria Academic Hospital. The umbilical cords used during the isolation process had to be collected from the theatre as soon as possible following the caesarean section. Only the umbilical cord collected from caesarean sections could be used for HUVEC isolation because normal birth is not considered a sterile procedure and could potentially cause contamination of the isolated cell cultures. Once the placenta and umbilical cord had been obtained, the umbilical cord was carefully inspected and a section of approximately 20 cm long, free from any clamp marks or blood clots was cut and prepared for further use. The placenta and the rest of the umbilical cord was disposed of in a human tissue-disposal container in the theatre. The selected section of umbilical cord was gently cleaned with sterile saline+ solution. The clean umbilical cord made it easy to distinguish between the larger vein and the two

smaller arteries (Figure 2.1). One side of a small sterile plastic tube (2 mm diameter) was inserted into the vein on one side of the cord. The other side of the tube was then connected to a 20 ml syringe filled with saline+. The saline+ solution was then slowly and gently flushed through the vein to remove residual blood. This step was done in the theatre to ensure blood removal before coagulation occurs. To ensure the best results, all the residual cord blood was flushed from the vein and the outside of the umbilical cord was rinsed thoroughly with saline+. The clean umbilical cord was placed in a sterile container and submerged in saline+ and stored at 4^{o}C for a maximum period of 5 hours before use (Figure 2.3).

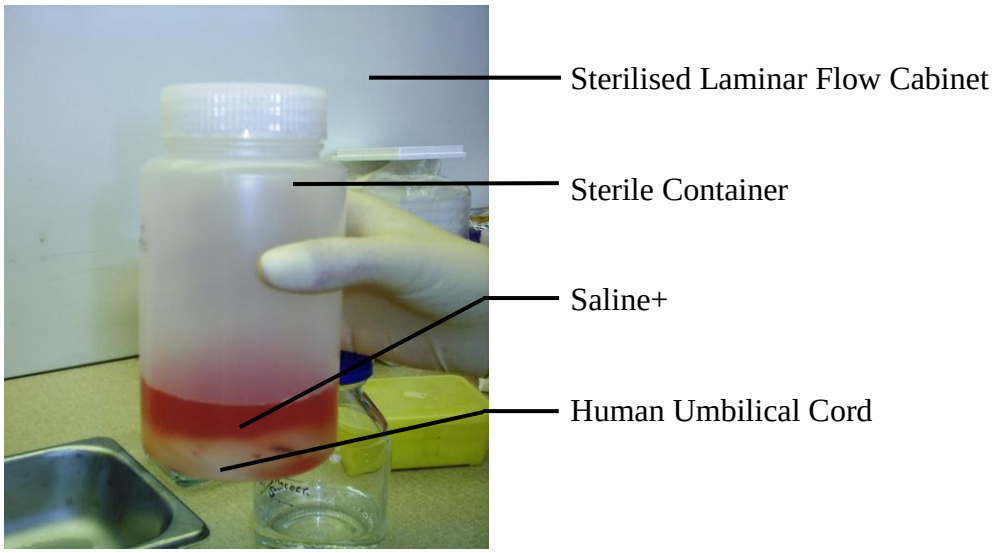


Figure 2.3. A photograph of a human umbilical cord submerged in sterile saline+ and sealed in a sterile container.

ii. Procedures in the laboratory

Isolation of HUVEC and the preparation of HUVEC for the cell culture.

A water bath was preheated to 38^{o}C (optimal temperature for collagenase type I digestion) before starting the isolation procedure. The umbilical cord was removed from the saline+ and placed on a flat sterile surface. A sterile butterfly needle was placed into the opening of the vein on the one side of the umbilical cord and sealed with a small steel clamp. A 20 ml syringe, filled with HBSS was connected to the butterfly needle and used to gently flush the vein (Figure 2.4). It is essential to use HBSS without Ca^{++} and without

Mg^{++} as these ions result in severe damage to the HUVEC and a negligible number of cells survive the isolation procedure. The HBSS was flushed through the vein slowly and gently to avoid stretching or damaging of the vein.

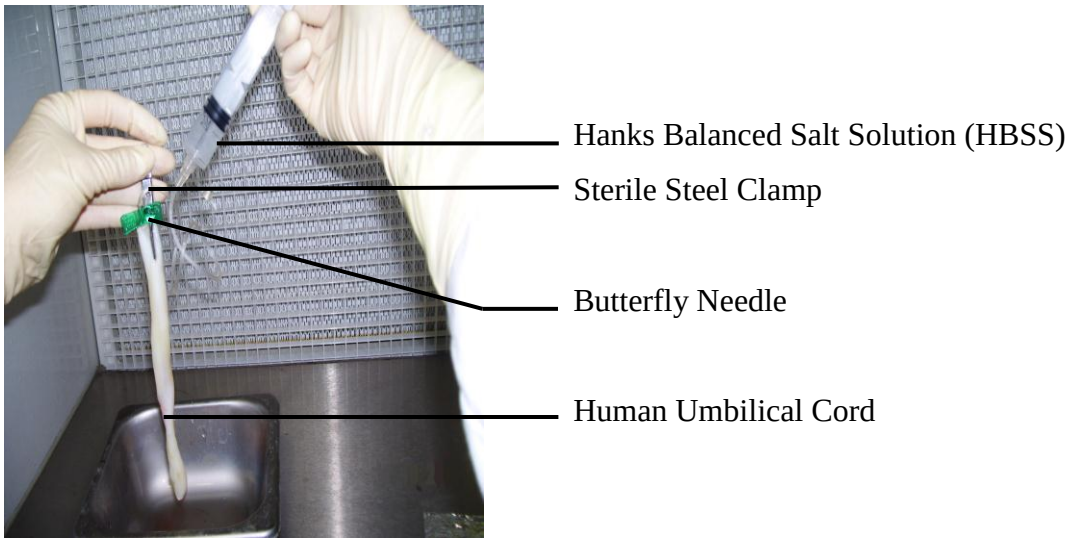


Figure 2.4. A photograph of a butterfly needle placed into the one side of a human umbilical cord vein. The vein opening is sealed with a steel clamp and HBSS is gently flushed through the vein with a syringe.

The vein was left to drain by gravity before injecting a 1 mg/ml collagenase type 1 solution. The endothelial vein cells were released from internal surface of the vein by this enzymatic digestion (initial experiments indicated that collagenase I produced better HUVEC than collagenase type IV). The collagenase was injected into the vein using the 20 ml syringe until a few drops of the collagenase exited the vein on the opposite side. The end of the vein was then sealed with a sterile steel artery clamp. The vein was filled with collagenase (± 10 ml) and placed onto the flat sterile surface. Slight pressure was exerted onto the umbilical cord using the palm of the hand (with sterile gloves) followed by a rolling movement. This movement was performed for approximately 20 seconds (Figure 2.5).

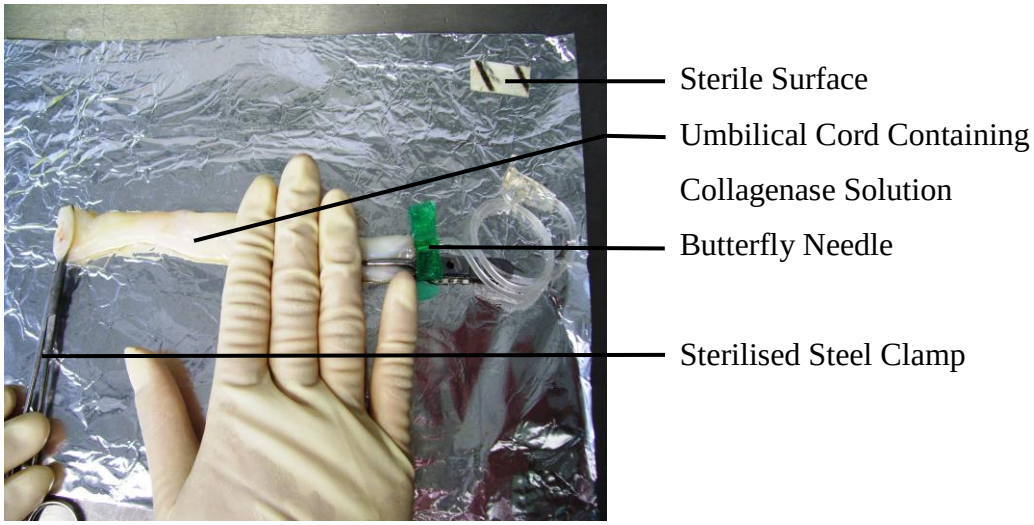


Figure 2.5. A photograph of a rolling movement performed on a human umbilical cord directly after collagenase was injected into the vein.

The umbilical cord was then placed, suspended by its two ends, into a container filled with pre-heated PBS-. This container was placed into the water bath and the umbilical cord was incubated at 38^{o}C for 15 minutes (initial experiments indicated that 15 minutes at 38^{o}C produced the best results). After incubation the umbilical cord was removed from the PBS- and the collagenase solution (now containing free endothelial cells) was allowed to drain directly into a sterile centrifuge tube containing 15 ml of 199 medium+. The remaining collagenase solution was gently flushed from the vein using 199 medium+ (± 10 ml) and the effluent was collected into the sterile centrifuge tube containing the collagenase solution and medium (Figure 2.6). The umbilical cord was disposed of in a human tissue-disposal container.

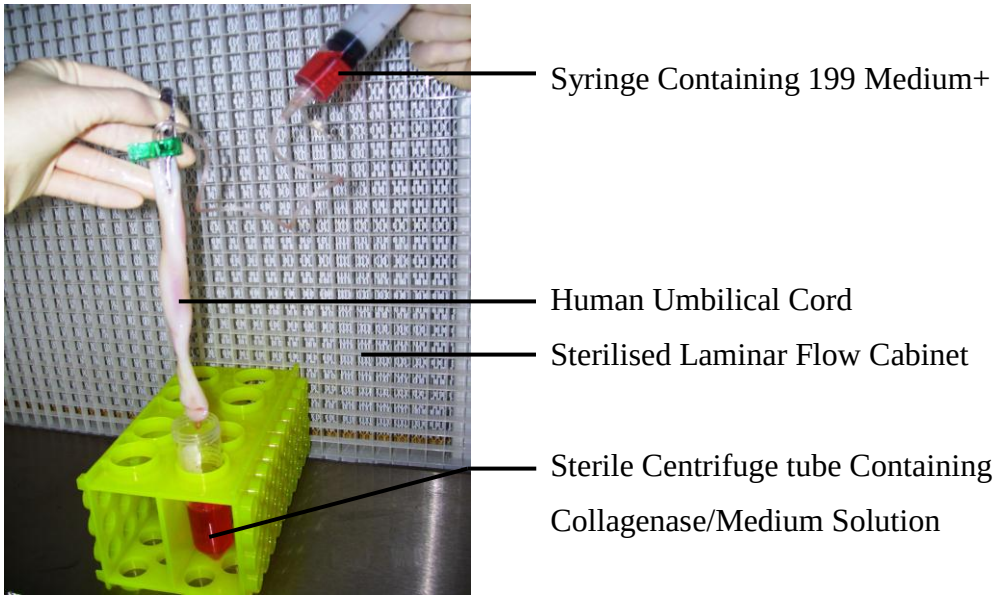


Figure 2.6. A photograph of a human umbilical cord vein flushed with 199 medium+ to remove remaining collagenase solution.

The collagenase solution containing the cells was centrifuged at 200 *g* for 7 minutes (Figure 2.7).

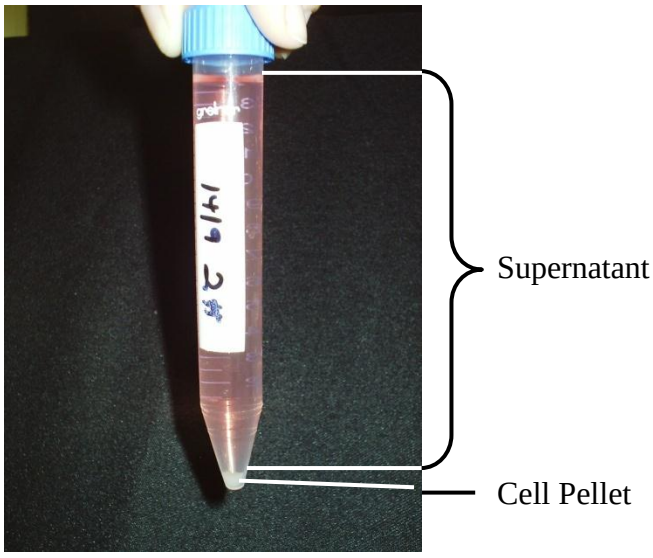


Figure 2.7. A photograph following centrifugation (7 minutes at 200 *g*) of a 15 ml tube containing a collagenase/cell solution. The HUVEC pellet can clearly be distinguished from the supernatant (199 medium and collagenase solution).

After centrifugation, the supernatant was removed from the tube using a sterile Pasteur pipette without disturbing the pellet (endothelial cells). The cell pellet was washed by adding 15 ml of 199 medium+ to remove the last traces of collagenase from the pellet. Damage to the endothelial cells could occur if the cells are exposed to collagenase for extended periods of time. The solution was centrifuged at 200 *g* for 7 minutes for a second time, the supernatant removed and the pellet resuspended into 15 ml of 199 medium+ before adding 75 µl of endothelial cell growth factor (ECGF) and 75 µl of heparin and transferring it to an appropriate tissue culture flask pre-coated with 1% gelatine (25 cm^2 flask). The combination of additives was used as the heparin increases the affinity of ECGF for its receptor through conformational change in the ECGF receptor (Konkle *et al*., 1988).

Gelatine coating of tissue culture flasks and tissue culture plates

The 1% gelatine solution was removed from the refrigerator and equilibrated to room temperature. The gelatine solution was sterilised with a 0.2 µm Sartorius cellulose acetate filter before every use. The gelatine solution was placed into a sterile tissue culture flask or tissue culture plate with the use of a sterile Pasteur pipette. The amount of gelatine solution used varied with different sizes of flasks and plates used. The bottom of the flask/plate had to be covered at least 1 mm deep with the gelatine solution and incubated for 90 minutes in a 37^oC at 5% CO_2 incubator. After the incubation period, a thin layer of gelatine attached to the bottom of the flask/plate and the excess gelatine solution was removed with a sterile Pasteur pipette and the flask/plate was rinsed with 199 medium+. The flask/plate was left to dry overnight before it was used to incubate the cells.

Incubating HUVEC

During incubation, the cells were examined daily under an inverted phase-contrast microscope to evaluate the adherence, proliferation and confluence of the cells (Figure 2.8). The 199 medium+ was changed every 48 hours. At 80% confluence, most of the cells had an epithelioid morphology and was associated into colonies (Figure 2.10 (d)). The time required to reach 80% confluency depended on the starting cell density.

The cells were trypsinised and passaged at 90-95% confluence to prevent overgrowth.

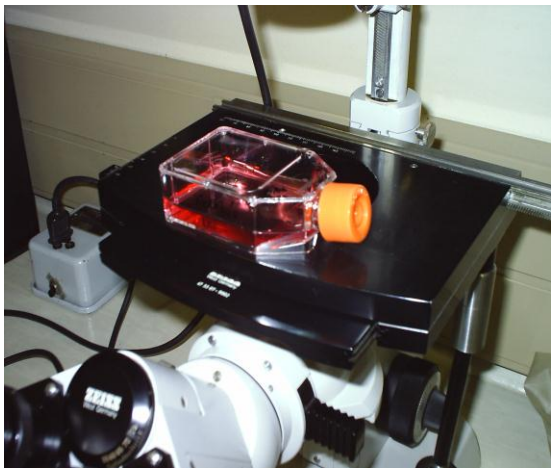


Figure 2.8. A photograph of a 25 cm^2 culture flask containing HUVEC and 199 medium+.

Trypsinising of HUVEC

The medium of the selected culture flask was aspirated before trypsinising. The monolayer of cells was briefly rinsed with ± 3 ml trypsin solution to remove excess medium. After rinsing the monolayer was covered with fresh trypsin solution, 2-3 ml for a 25 cm^2 flask or 5-6 ml for a 75 cm^2 flask and allowed to incubate at 37^oC for approximately 3-5 minutes (cells detach from the bottom of the flask and trypsin solution appears translucent). Excessive exposure of cells to trypsin should be avoided to prevent cell damage. Cells were examined under the microscope to determine whether the cells had detached and if so, collected into a sterile 15 ml centrifuge tube. The tube was filled with 199 medium+ and centrifuged at 200 *g* for 7 minutes to harvest the cells. After centrifugation, the supernatant was removed and discarded. The pellet was resuspended in 1 ml 199 medium+, the solution was divided into two gelatine coated 75 cm^2 culture flasks, 30 ml of 199 medium+ was added to each of the flasks and the flasks were placed into the CO_2 incubator (Figure 2.10 (a)).

HUVEC were used for experiments at their optimal viability (2nd and 3rd passage) and were discarded after the 4th passage.

Freezing of tissue culture cells

This procedure was done after trypsinising the cells. The tube containing the cell pellet (obtained during the trypsinising procedure) was placed on ice to keep the cells chilled and thus slowing their metabolism and preventing clumping. The cell pellet was suspended in 1 ml freeze media (as prepared in section 2.3.1.xi) and mixed gently but thoroughly using an automatic pipette. The suspension was transferred into cryotubes (1.8 ml volume vial). The cryotubes were placed into polystyrene foam boxes and stored at -70^{o}C for at least 3 days. The cryotubes were then speedily transferred to liquid nitrogen to avoid warming of the vials. Frozen cells were stored in liquid nitrogen until needed.

Thawing of tissue culture cells.

The vials containing the frozen HUVEC were removed from the liquid nitrogen and placed in warm water until completely thawed (60-90 seconds at 37^{o}C provides best recovery). After thawing the cells, the cell suspension was added to 15 ml of 199 medium+ pre-heated to 37^{o}C. This mixture was centrifuged for 7 minutes at 200 *g* (to separate the cells from the freeze medium), the supernatant (freeze medium) discarded and the pellet (cells) resuspended in fresh 199 medium+. The new cell suspension was transferred to a 1% gelatine coated 25 cm^{2} tissue culture flask and placed into a 5% CO_2 incubator at 37^{o}C. The medium was replaced after 24 hours of incubation (Figure 2.10 (c)).

2.4. Results

Several human umbilical cords were used during this study and Figure 2.9 represents an example of a clamp and blood clot free part of a human umbilical cord selected for HUVEC isolation.

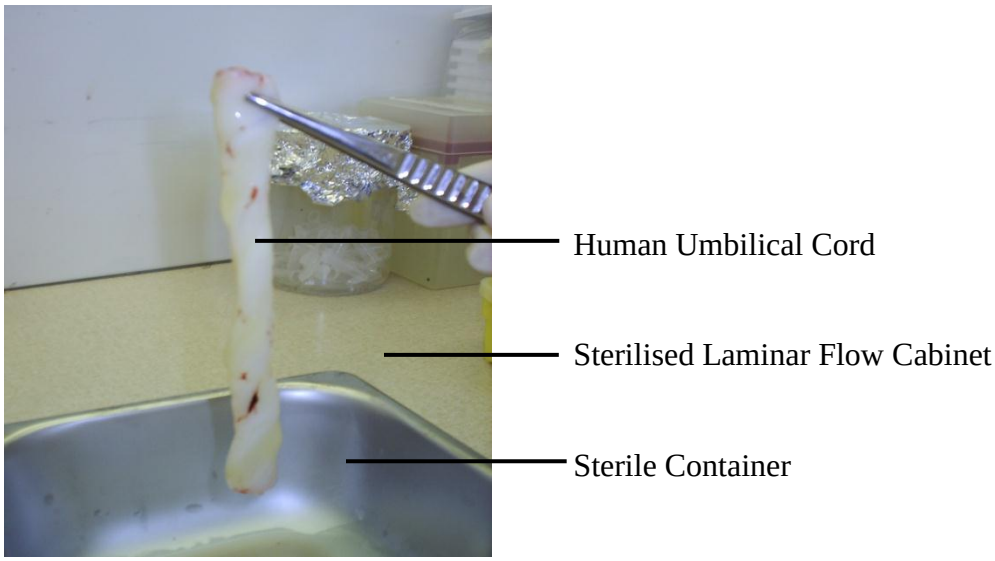
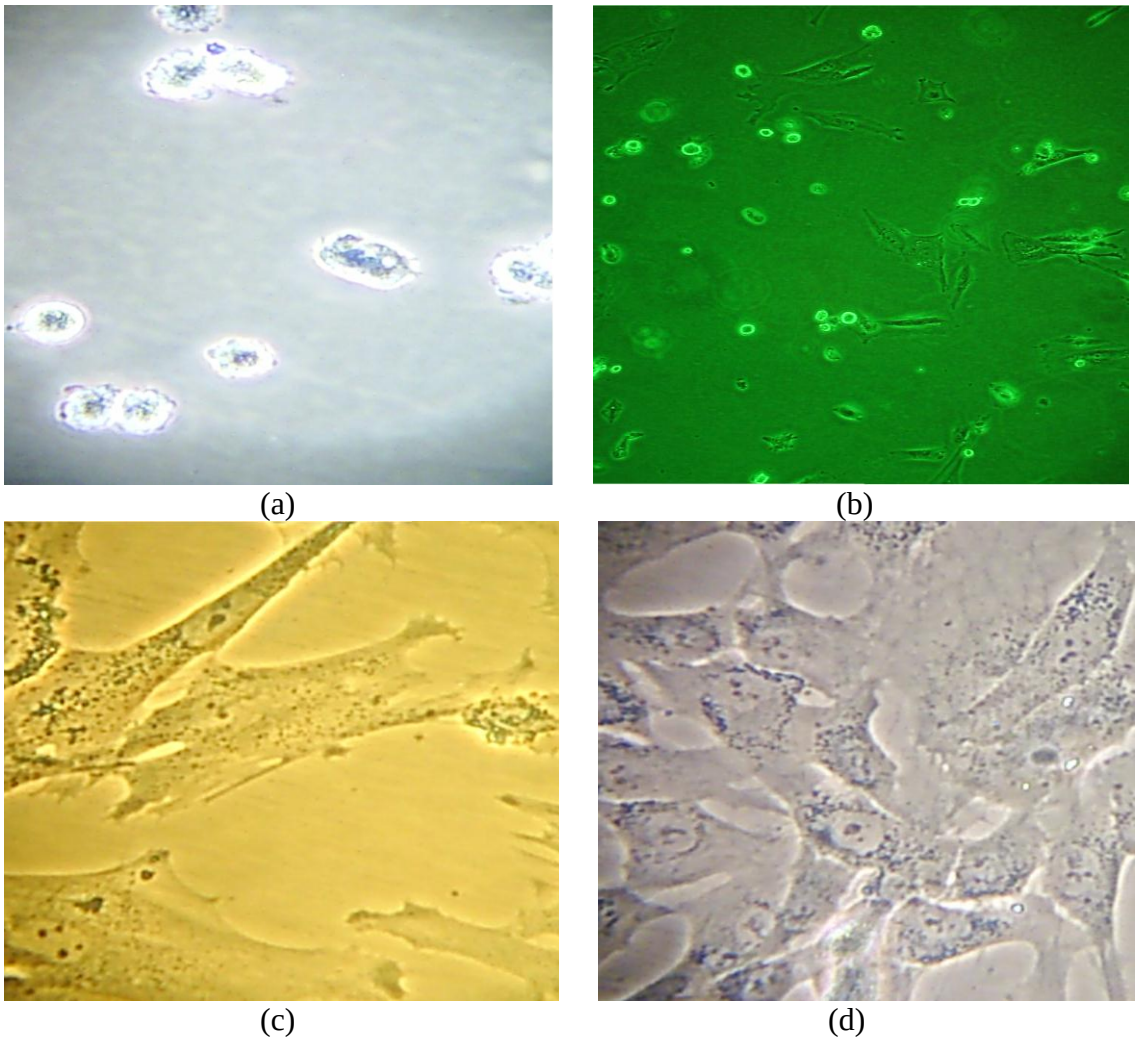


Figure 2.9. A photograph of a selected part of a human umbilical cord free from blood clots and clamp marks.

The isolation procedure, trypsinising, freezing away and thawing methods used during this experiment produced healthy HUVEC in sufficient numbers for further use. Directly after the isolation procedure, trypsinising procedure or thawing procedure, the HUVEC have a rounded shape when viewed under a microscope (Figure 2.10 (a)). This is due to the cleavage of proteinacious cell surface adhesion factors caused by the proteolytic effects of trypsin and the fact that the cells are not attached to a surface to counter the cytoskeleton. Approximately 6 hours after these procedures (isolation, trypsinising, or thawing), the viable cells will attach to the surface of a gelatine coated culture flask (Figure 2.10 (b)). The cell shape will change to a flat polygonal cobblestone shape as the HUVEC proliferates (Figure 2.10 (c) and (d)). The HUVECs were never allowed to exceed 95% confluence before using for experimental procedures or dividing the cultures. Overgrowth of the cells reduced their viability and negatively influenced their further proliferation.



(a) (b)

(c) (d)

Figure 2.10. Photographs taken during this study of the HUVEC culture at different stages of proliferation at 20x magnification.

(a) HUVEC directly after isolation or following trypsinising
(b) HUVEC 6 hours after isolation procedure/trypsinising
(c) 24 hours after isolation procedure/trypsinising
(d) 80-95% confluence.

2.5. Discussion

The purpose of this phase of the study was to establish a usable technique to isolate human umbilical vein endothelial cells (HUVEC) from fresh human umbilical cord within the local laboratory and to determine suitable culture conditions for these cells. The development of a suitable culture environment for HUVEC was important, because of the extensive use of these cells during further experimentation.

HUVEC was an ideal cell type to use for anti-inflammatory drug evaluation due to its ability to produce cytokines, its ability to express adhesion molecules such as ICAM-1 and its ability to facilitate the adhesion of neutrophils to the endothelium. HUVEC was therefore selected in light of the important roll endothelial cells play in the inflammatory response and the documented presence of cannabinoid receptors on the surface of these cells (Liu *et al.*, 2000).

The health, viability, shape and morphology of the HUVEC was visually checked using a microscope at 100x and 400x magnification to assess the success of the different methods. Cell proliferation should not be the only indicator of healthy and viable cells, making visual analysis essential. Monitoring cell population, cell density, degree of confluence, shape as well as attachment to the culture flask impacted the quality of primary culture produced. HUVEC primary cultures have specific characteristics that indicate health status and due to this, visual analysis was preferred.

During the experimentation with different techniques of isolation and different conditions for culturing HUVEC, several crucial points were determined i.e.

- It is essential to use HBSS and PBS without Ca^{++} and without Mg^{++} because these ions cause severe damage to the HUVEC and an insignificant number of cells survive the isolation procedure.
- It is important to rinse the collagenase from the umbilical cord using 199 medium+ instead of HBSS during the isolation procedure. A comparison of the two different methods indicated that less damage occurred to the isolated HUVEC when 199 medium was used to flush the remaining collagenase (containing the cells) from the umbilical cord.
- Collagenase type I released more endothelial cells by enzymatic digestion than collagenase type IV.
- One milligram per millilitre of collagenase type I in HBSS produced the best results. Higher concentrations of collagenase produced a cell population consisting of HUVEC, muscle cells and fibroblasts. Fibroblasts proliferated more

rapidly than HUVEC and swamped HUVEC proliferation after the 2nd passage resulting in primary fibroblast cultures.

- Incubating the collagenase in the umbilical cord for 15 minutes at 38°C produced notably more HUVEC from the isolation procedure when compared to 15 minutes at only one degree centigrade less ie 37°C.
- It was found that the isolated or thawed HUVEC proliferate faster when the culture medium was completely replaced 6 hours after the isolation or thawing procedure.
- It is imperative to use cell culture tested gelatine as opposed to standard non-culture tested gelatine. HUVEC proliferate faster on cell culture tested gelatine.

The optimal growth medium to use during HUVEC culturing was found to be 199 Medium supplemented with 20% FCS, 75 µl ECGF, 75 µl heparin and 2 mM glutamine. ECGF combined with heparin significantly increased the proliferation of HUVEC. These findings were in keeping with the results obtained by Terramani *et al.* (2000). According to Terramani *et al*. (2000), 199 Medium is the most frequently used culture medium for HUVEC.

2.6. Conclusion

Human primary cell cultures are often selected for mechanistic studies, because it is thought to better represent the *in vivo* situations and generate more physiologically relevant data. Although primary cells have a limited lifespan, given the right conditions these cells can be of great value. Cell isolation procedures and culture conditions vary widely for each cell type and adjusting the method for HUVEC isolation and culturing was vital to ensure the success of the research project.

A suitable technique for isolating and the conditions for culturing of HUVEC within the local laboratory were established. The final method used to isolate and culture HUVEC was similar to previous methods used in other publications, but was modified and tweaked by incorporation different finding from several publications to produce primary cultures in the laboratory where the research was carried out. These methods supplied

flat polygonal cobblestone shape HUVEC cultures with all the visual characteristics of healthy, viable cells to be used in further experimentation for the evaluation of the anti-inflammatory properties of Rimonabant.

These methods were used throughout the rest of the study to isolate and culture healthy HUVEC for use to determine anti-inflammatory potential of Rimonabant and anandamide.

Chapter 3: Evaluation of cell growth inhibition

3.1. Introduction

In this chapter, the cytotoxicity of Rimonabant and anandamide will be determined. The aim of this phase of the study was to establish the concentration at which the two selected compounds (Rimonabant and anandamide) are toxic to HUVEC as well as to human lymphocytes. It is essential to verify the concentrations that can be used in further experimentation without any signs of cytotoxicity which would be a confounding factor. In order for a drug to be effective as an anti-inflammatory treatment, it has to exert anti-inflammatory effects at concentrations that are non-toxic to normal human cells.

The intention of *in vitro* measurements of cell proliferation or survival is to obtain an estimation of the percentage of cells that have entered the cell cycle (as evidenced by DNA synthesis), the amount of cell division or a decrease in overall cell numbers as a result of compound exposure or specific culture conditions.

This study objective required an assay to measure the survival of cells treated with different concentrations of Rimonabant and anandamide. A colorimetric assay making use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), was selected for this experiment. This method is one of the most commonly used methods to assess cytotoxicity and cell proliferation (Molinari *et al*., 2005). According to Mosmann (1983), who tested several tetrazolium salts with different cells to determine the best assay, MTT was found to produce the most promising results. MTT is a light sensitive, water-soluble yellow substrate that produces a dark blue/purple water-insoluble formazan crystallization product when incubated with living cells (Figure 3.1).

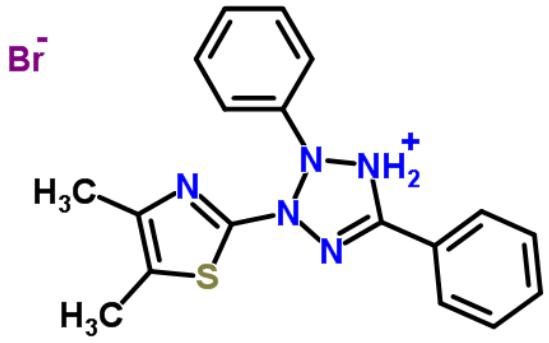


Figure 3.1. A structural representation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Barltorp *et al*., 1991).

The MTT formazan product is essentially insoluble in medium and it is thus necessary to use DMSO to dissolve the formazan in order to produce a homogeneous solution suitable for measurement. Once the formazan crystals are dissolved in DMSO, quantification of the coloured product offers a relative estimate of cellular viability and metabolic activity. The results obtained from this MTT colorimetric assay is measured by an automatic microplate scanning spectrophotometer (Mosmann, 1983).

Exposing cells to high concentrations of a cytotoxic compound is likely to kill a significant percentage of the living cells. The staining assay selected for cell enumeration should thus selectively quantitate only living cells and not dead cells in order to be effective. The membrane-permeable tetrazolium salt, MTT, has been demonstrated to actively colour live cells and have no staining effect on dead cells, even immediately after cell death. Once the MTT is inside the cell it is reduced to a formazan product in the mitochondria. The MTT assay is thus based on the capacity of the cell to convert the MTT substrate to a formazan in its mitochondria. The ability of the cell to convert MTT is thus directly proportional to the general metabolic state of the tested cell (Hynes *et al*., 2006). Cell number can therefore be measured by examining the intensity of the purple colour produced by the dissolved formazan crystals (Figure 3.2). A large number of living cells or cells with a higher metabolic activity (stimulated cells) will produce more formazan, hence a more intense colour, than a smaller number of cells or resting cells would do (Figure 3.2).

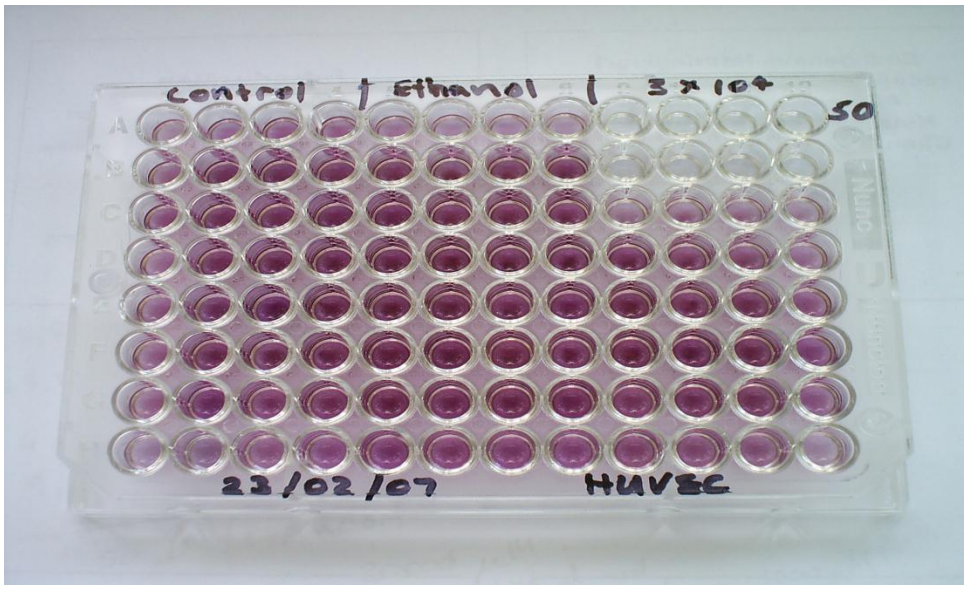


Figure 3.2. A photograph of a typical 96 well tissue culture plate containing HUVEC (3 x 10^4 cells per well) as used to determine the cytotoxicity of Rimonabant (serial dilutions starting from 50 µM) and ethanol (serial dilutions starting from 10% ethanol). These wells were treated with MTT and re-incubated for 4 hours before the purple formazan was dissolved in DMSO.

Mosmann (1983) presented several advantages to using the MTT colorimetric assay making it the preferred assay to use during proliferation of cytotoxicity assays. These advantages include:

- There is no radioactivity involved in this assay
- Adherent cell lines, as well as cells in suspension can be used in this assay
- It's a fast and easy method to follow
- High-throughput analysis using a scanning multiwell spectrophotometer

It is thus clear that the MTT assay was a suitable method for determining the cytotoxicity of Rimonabant and anandamide on HUVEC as well as on human lymphocytes.

3.2. Aim

The aim of this study was to determine the maximum *in vitro* concentrations at which Rimonabant and anandamide have insignificant cytotoxic effect on selected human cells and in doing so, determine suitable concentrations for further experimentation. This was accomplished by following the following steps:

i. Determine the cytotoxicity of various ethanol concentrations on HUVEC (control for the ethanol used to dissolve Rimonabant and anandamide).
ii. Determine the cytotoxicity of Rimonabant or anandamide on HUVEC.
iii. Determine the cytotoxicity of Rimonabant or anandamide on human lymphocytes.
iv. Determine whether Rimonabant used in combination with anandamide demonstrated any synergistic cytotoxic effect on human lymphocytes.

3.3. Materials and Methods

The method (MTT assay) used to determine cytotoxicity of Rimonabant, ethanol and anandamide was previously described by Mosmann (1983) and modified by Van Rensburg (1996) to use DMSO instead of acid-isopropanol to dissolve the formazan crystals. Rimonabant and anandamide were prepared by dissolving the compounds in 100% ethanol. Ethanol was thus included in the cytotoxicity study to ascertain the possible involvement of ethanol in the cytotoxicity of the cells. Ethanol was tested at 8 different concentrations i.e. 0.078%, 0.156%, 0.313%, 0.625%, 1.25%, 2.5%, 5% and 10%. This ethanol experiment was repeated 5 times on HUVEC using 5 different donors.

The volunteers used in this study were carefully selected according to certain inclusion and exclusion criteria to ensure healthy lymphocytes for the experiment (refer to Section 3.2). Each patient was required to sign an informed consent form verifying their authorization for the use of their blood during this research project (refer to Annexure C).

3.3.1. Preparation of experimental compounds.

The prepared compounds were sterilised before use and diluted, if needed, with sterile solutions. The sterile compounds were stored in an airtight sterile container and only opened inside a sterilised laminar flow cabinet (Labotec, Model LCA 6000VA) to prevent contamination.

i. Rimonabant (Acomplia/SR 141716A)

Rimonabant was a generous gift from Sanofi Aventis.

Molecular weight: 463.8 g/mole

A stock solution of 20 mM was prepared by dissolving 9.3 mg of Rimonabant in 1 ml of 100% ethanol. The stock solution was stored in 50 µl aliquots at –20°C. The relevant dilutions were made with the appropriate tissue culture medium prior to each experimental procedure.

ii. Anandamide (AEA/Arachidonylethanolamide)

Anandamide was obtained from Sigma Aldrich (St Louis, USA).

Molecular weight: 347.53 g/mole

A stock solution of 20 mM was prepared by dissolving 6.9 mg of anandamide in 1 ml of 100% ethanol. The stock solution was stored in 50 µl aliquots at –20°C. The relevant dilutions were made with the appropriate tissue culture medium prior to each experimental procedure.

3.3.2. Preparation of reagents

All the prepared reagents were sterilised before use and placed into a sterile airtight container for storage. The sterile container was only opened inside a sterilised laminar flow cabinet to prevent possible contamination. 199 Medium+, gelatine, heparin and DMSO were prepared according to the method described in Section 2.3.1.

i. Phytoheamagglutinine (PHA)

PHA was purchase from Bioweb (Edenglen, Johannesburg, RSA). The freeze-dried content of one vial was mixed with 5 ml sterile distilled water and dispensed into 0.2 ml aliquots, which were stored at -20°C. Upon use, 0.8 ml medium was added to 0.2 ml PHA to provide a 1:4 dilution for use in experimental procedures (final concentration 2.5 µg/ml).

ii. Phosphate Buffered Saline+ (PBS+)

PBS was obtained from The Scientific Group (Johannesburg, RSA). 9.23 g of PBS powder was dissolved in 1000 ml of distilled water and the pH of the solution was adjusted to 7.2 using HCl or NaOH. This solution was stored at 4°C.

iii. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) stain solution

MTT, was purchase from Sigma Aldrich (St Louis, USA). 200 mg of MTT was dissolved in 40 ml of PBS and sterilized with a 0.2 µm pore size syringe filter. This MTT solution was stored at 4^{o}C in the dark (the stain is light sensitive).

iv. Histopaque

Sterile Histopaque was purchased from Sigma Aldrich (St Louis, USA) and used undiluted.

v. RPMI^{+}

RPMI-1640 was purchased from Sigma Aldrich (St Louis, USA). 52 g RPMI-1640 was dissolved in 5000 ml of autoclaved ultra-pure, pyrogen-free, de-ionized water. De-ionized water was produced by an in-house Elga PureLab Ultra water unit. 10 g $NaHCO_3$ (Sigma) was added just before filtration to adjust the pH. The pH was adjusted further, if necessary, by adding small quantities of 1 N HCl or 1 N NaOH until a pH of 7.4 was reached. The medium was filtered by using a Sartorius vacuum flask filter system with a Sartorius glass-fibre pre-filter and a 0.2 µm Sartorius cellulose acetate filter. The medium was filtered a second time using two Sartorius glass-fibre pre-filters, which preceded the sterile Sartolab P pressure filtration unit (0.2 µm). A Heidolph peristaltic pump was used to force the medium through the filters. The medium was dispensed into sterile 500 ml flasks containing 1% P/S. The medium was stored at 4^{o}C until needed. The medium was supplemented with 10% FCS just before use (RPMI^{+}).

vi. Ammonium chloride

All the reagents were supplied by Sigma Aldrich (St Louis, USA).

8.3 g Ammonium chloride (NH_4Cl)

1 g Sodium bicarbonate ($NaHCO_3$)

74 mg Ethylenediamine tetraacetate (EDTA)

The reagents were dissolved in 1000 ml distilled water and filter sterilised into a sterile container. The ammonium chloride was stored in a cold room or a refrigerator (4^{o}C).

vii. Cell counting fluid

One millilitre of a crystal violet solution (0.1%) obtained from Sigma Aldrich (St Louis, USA,) and 2 ml of acetic acid obtained from Merck (Darmstadt, Germany) were added to 97 ml distilled water, mixed well and refrigerated (4^{o}C).

3.3.3. Experimental procedures

i. Lymphocyte preparation

Venous blood was collected from a healthy volunteer and heparinised (5 units heparin/ml blood) to prevent the blood from clotting. In a 50 ml centrifuge tube, 35 ml of the heparinised blood was carefully overlaid onto 15 ml Histopaque with a Pasteur pipette and centrifuged for 25 minutes at 650 *g* (at room temperature). This was done to separate the different cell types present in human blood. The top plasma layer was discarded and the lymphocyte/monocyte band (± 12 ml) was transferred to a sterile 50 ml centrifuge tube, which was then filled with sterile RPMI-1640 medium. The solution was centrifuged for 15 minutes at 200 *g* to separate the contaminating platelets from the lymphocytes. The supernatant fluid was discarded and the pellet was resuspended in 50 ml sterile RPMI-1640 medium. The cell suspension was centrifuged again for 10 minutes at 200 *g* (this step was done to ensure that any contaminating platelets were removed). The supernatant was discarded and the pellet was resuspended in 50 ml sterile, cold ammonium chloride and left to stand on ice for approximately 10 minutes. This step was done to lyse any contaminating red blood cells present in the cell suspension. The cell suspension was centrifuged for 10 minutes at 200 *g*, the supernatant was discarded and the pellet was resuspended in 1 ml RPMI^{+}. A 50 µl aliquot of the cell suspension was counted with cell counting fluid and diluted in RPMI^{+} to 2 x 10^{6} cells/ml RPMI^{+}. This was the concentration of lymphocytes used during the proliferation experiments. The prepared lymphocytes were used in experiments immediately and could not be stored for later use.

ii. HUVEC preparation

HUVEC flasks were trypsinised as described in Chapter 1 and the washed pellet was resuspended in 1 ml 199 Medium+. The cell suspension was mixed thoroughly by pipetting repeatedly with an automatic pipette to make sure that the cells were separated from each other and that a uniform suspension was formed. The cells were counted using cell counting fluid and diluted in 199 Medium+ to a concentration of 3×10^4 cells/ml 199 Medium+. Sterile 96 well tissue culture plates were pre-coated with 1% gelatine before use with HUVEC.

iii. Counting of the cells

With the use of an automatic pipette, 50 µl of the cell suspension (prepared as described above) was added to 450 µl cell counting fluid and mixed well (the tube containing the suspension was lightly swirled for at least 2minutes). A small volume (approximately 20 µl) of this suspension was placed under the coverslip of a haemocytometer and the cells were counted under a microscope at 100x magnification. In the case of large numbers of cells, only 5 of the 25 blocks were counted: four in the corners and the middle block. For example if 5 blocks were counted and the result was 100 cells, it would be 100 x 5 = 500 cells on the counting chamber. The constant of the counting chamber is 1×10^4. The dilution made to count the cells in counting fluid is 1:9 dilution, therefore the number of cells counted is: $500 \times 10 \times 10^4 = 500 \times 10^5$.

iv. Cell proliferation assay

The sterile 96 well tissue culture plates were divided into sections representing the different drug concentrations as well as the control groups. Gelatine coated tissue culture plates were used for HUVEC whereas uncoated tissue culture plates were used for human lymphocytes. Tissue culture medium (199 Medium+ for HUVEC and $RPMI^+$ for lymphocytes) was dispensed (80 µl) into each of the 96 wells. Only 60 µl medium was dispensed into each well in the case of lymphocytes intended to be stimulated. The cell suspension was prepared (HUVEC = 3×10^4 cells/ml or lymphocytes = 2×10^6 cells/ml) and 100 µl was added to each well. The tissue culture plates containing the cell suspensions were incubated for 60 minutes in a 37°C/5% CO_2 incubator. After this initial

incubation period, 20 µl of the experimental drugs (various concentrations) (Figure 3.4, 3.5, 3.6, 3.7) was dispensed into the labelled wells and 20 µl of the tissue culture medium was dispensed into the untreated control wells. Lymphocytes intended to be stimulated received 20 µl PHA 5 minutes after the addition of the drugs. The final volume in each well was 200 µl. The tissue culture plates were placed into a sterile container along with a small amount of distilled water. This container was placed into a 37°C/5% CO_2 incubator and allowed to incubate for 5 days in the case of HUVEC and 3 days in the case of lymphocytes.

v. MTT assay

After the incubation period, as described above, 20 µl of the MTT solution was added to each well. The tissue culture plates were re-incubated for approximately 3½-4 hours at 37°C/5% CO_2. After incubation, the plates were centrifuged for 10 minutes at 800 *g*, the supernatant was carefully removed with a Pasteur pipette and the cell pellets were washed by adding 150 µl PBS to each well. The plates were then centrifuged at 800 *g* for 10 minutes. The supernatant was again carefully removed without disturbing the pellets and the plates were placed into an Inco Therm^{e} Labotec oven (40^{o}C) for 1 hour to dry (plates can alternatively be stored in a dark place and left to dry overnight). The plates were dried completely to prevent any dilution of the DMSO. The diluted DMSO will in turn affect the intensity of the colour produced by the dissolved formazan crystals and in so doing affect the end results. Once the plates were dry, DMSO (100 µl) was dispensed into each well after which the plates were placed onto a shaker for approximately 1 hour. The absorbance was measured spectrophotometrically on a Universal Microplate Reader (Elx800 UV, Bio-Tek Instruments) using a wavelength of 570 nm and a reference wavelength of 630 nm.

The mean absorbance of the treated cells (at each concentration) was divided by the mean absorbance of the untreated controls and multiplied by 100 to calculate the percentage of control of cell growth in drug treated wells. These values were used to construct a survival curve from which the IC_{50} value was determined. IC_{50} value is the concentration (µM) of experimental compound inducing a 50% decrease in cell growth compared to the

untreated control. The IC_{50} values were calculated with the GraphPad Prism 4 statistics software programme.

3.4. Results

The amount of formazan produced by the drug (Rimonabant or anandamide) treated cells was compared to the formazan produced by the untreated control cells. The results are expressed as the mean percentage of untreated controls ± the standard error of the mean (SEM). Each assay was run in quadruplet per plate and repeated 5 times. All 5 repeats presented similar results. The results of these assays were pooled and an average value along with the SEM was calculated using the GraphPad Prism 4 software package. Both resting and stimulated lymphocytes were tested. The P-values indicate a comparison between the experimental values and the control values and were obtained by Mann-Whitney test. Kruskal-Wallis test was used to analyse the data obtained from the lymphocyte study.

The MTT assay was repeated 5 times using 5 different donors for each compound tested (Rimonabant, anandamide and ethanol) on HUVEC and was repeated 5 times (5 different donors) for resting and stimulated lymphocytes respectively as seen in Figures 3.3, 3.4, 3.5, 3.6 and 3.7. The MTT assay was then, in an independent experiment, repeated 5 times using 5 different donors to determine the cytotoxic effects of Rimonabant and anandamide at different concentrations in combination on human lymphocytes as seen below in Table 3.1.

The results obtained indicated that ethanol induced a toxic effect on HUVEC concentrations of 5% and higher (Figure 3.3).

Figure 3.4 and 3.5 represents the cytotoxicity curves of Rimonabant and anandamide respectively, when tested on HUVEC. Both Rimonabant and anandamide had dose related effects on the growth of HUVEC. The calculated IC_{50} values obtained after exposure of these cells to Rimonabant and anandamide were 14.3 µM and 124.2 µM respectively.

Figure 3.6 and 3.7 represents the cytotoxicity of Rimonabant and anandamide when tested on the growth of resting and stimulated human lymphocytes respectively. Rimonabant had a dose related effect on the growth of human lymphocytes. Anandamide had no effect on lymphocyte growth or survival at the highest concentration tested (25 µM). However, Rimonabant inhibited cell growth significantly at a concentration of 25 µM.

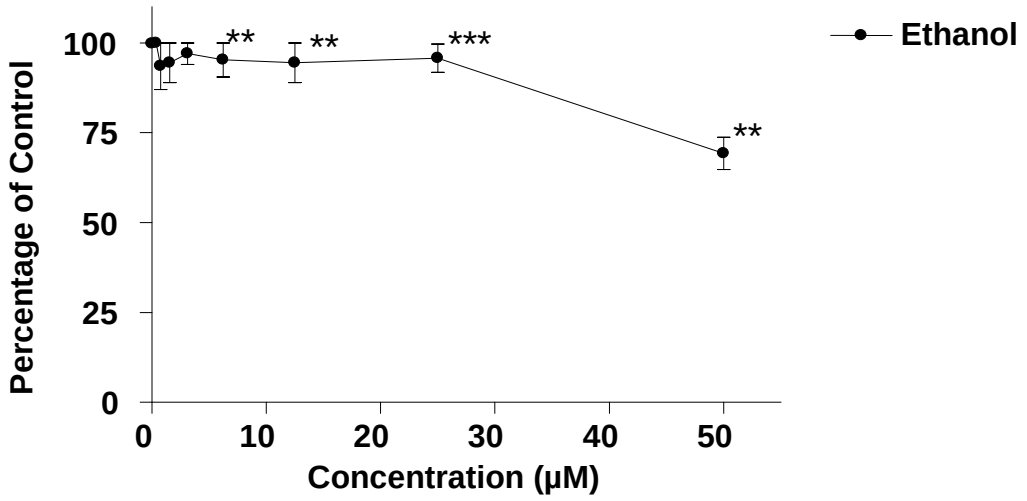


Figure 3.3. The mean HUVEC survival after 5 days of culture in the presence of various concentrations of ethanol.

*$p < 0.01$, **$p < 0.001$, ***$p < 0.0001$ compared to the untreated control

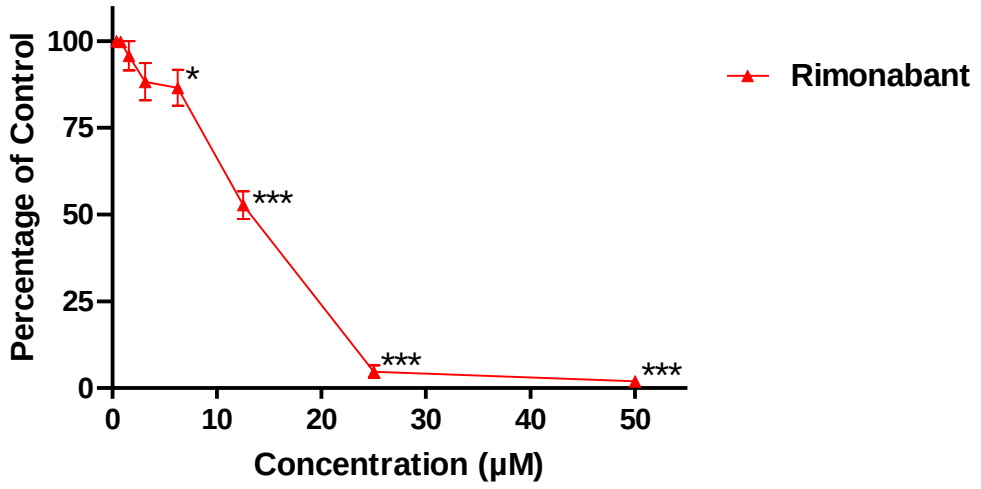


Figure 3.4. The mean HUVEC survival after 5 days of culture in the presence of various concentrations of Rimonabant.

*p < 0.01, **p < 0.001, ***p < 0.0001 compared to the untreated control

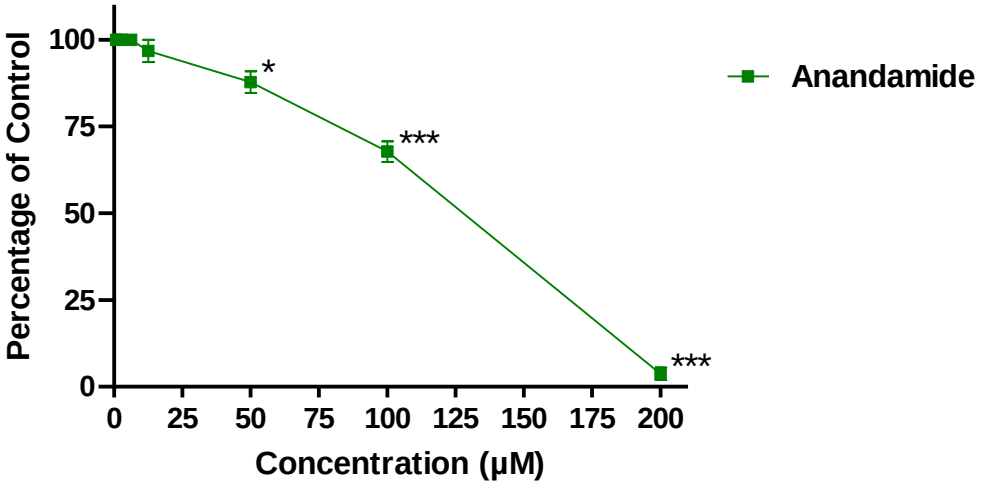


Figure 3.5. The mean HUVEC survival after 5 days of culture in the presence of various concentrations of anandamide.

*p < 0.01, **p < 0.001, ***p < 0.0001 compared to the untreated control

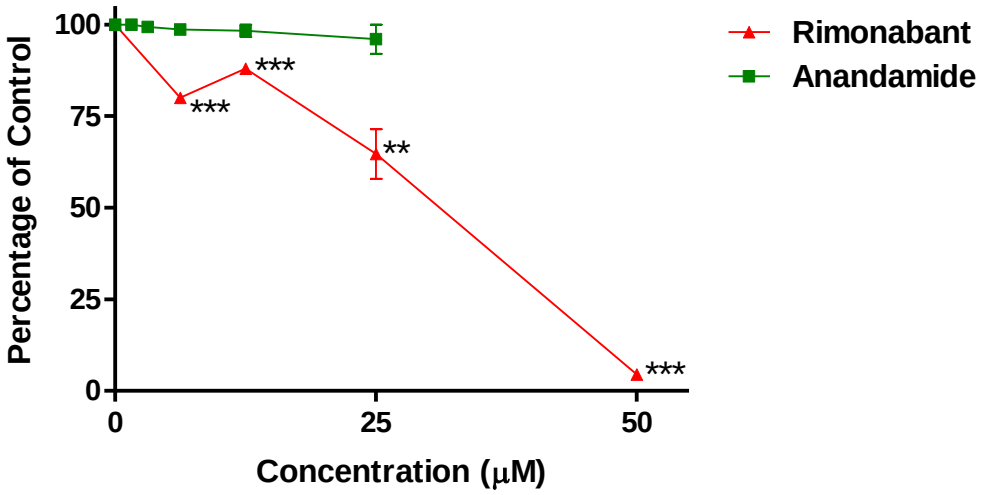


Figure 3.6. The mean percent resting lymphocyte survival after three days culture in the presence of various concentrations of Rimonabant or anandamide.

*p < 0.01, **p < 0.001, ***p < 0.0001 compared to the untreated control

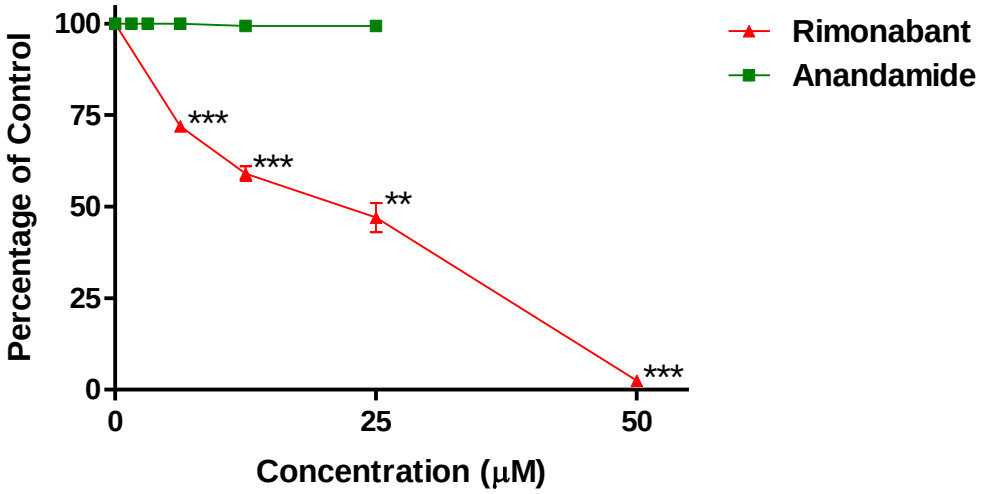


Figure 3.7. The mean PHA-stimulated lymphocytes survival after three days culture in the presence of various concentrations of Rimonabant or anandamide.

*p < 0.01, **p < 0.001, ***p < 0.0001 compared to the untreated control

Figure 3.8 and Figure 3.9 represents the data, expressed as a percentage of untreated control cells, obtained from lymphocyte cultures to determine whether Rimonabant and anandamide possess synergistic proliferative or anti-proliferative effects, when dosed together. Figure 3.8 represents the data from the resting human lymphocyte cultures whereas Figure 3.9 represents the PHA stimulated lymphocyte cultures. Synergistic anti-proliferative effects were observed in Figure 3.8 at the combination treatment of anandamide 6.25 µM and Rimonabant 25 µM for resting lymphocytes. In the case of PHA stimulated lymphocyte cultures, synergistic anti-proliferative effects were observed at the following combination treatments: Anandamide 12.5 µM / Rimonabant 25 µM and anandamide 25 µM/ Rimonabant 25 µM (Figure 3.9).

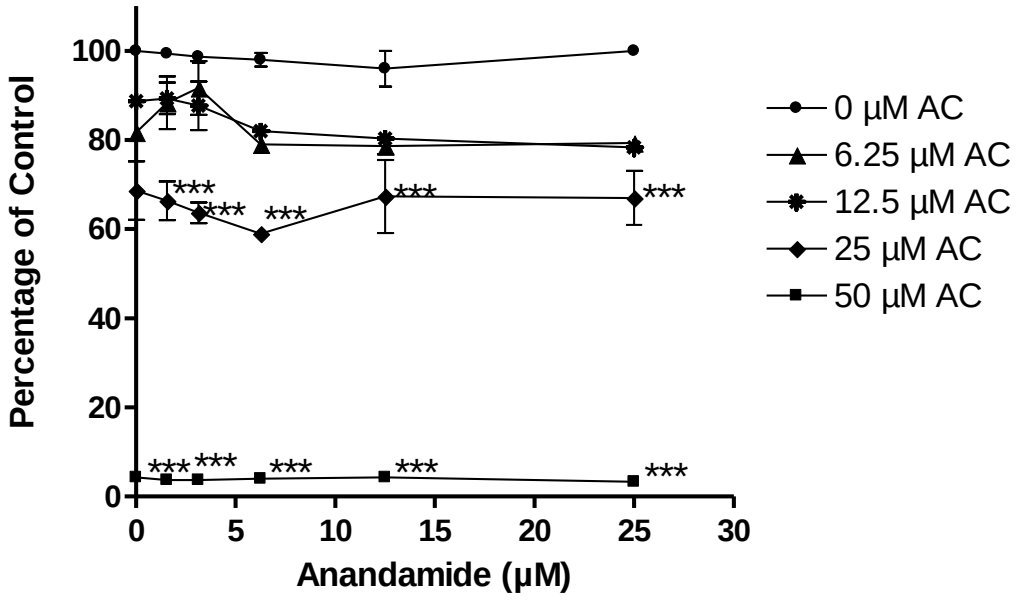


Figure 3.8. Percentage survival of resting lymphocyte cultures, after treatment with various concentrations of a combination of Rimonabant and anandamide. Results expressed as percentage survival of double treated cultures compared to the relevant untreated control values. *$p < 0.01$, **$p < 0.001$, ***$p < 0.0001$ compared to the untreated control

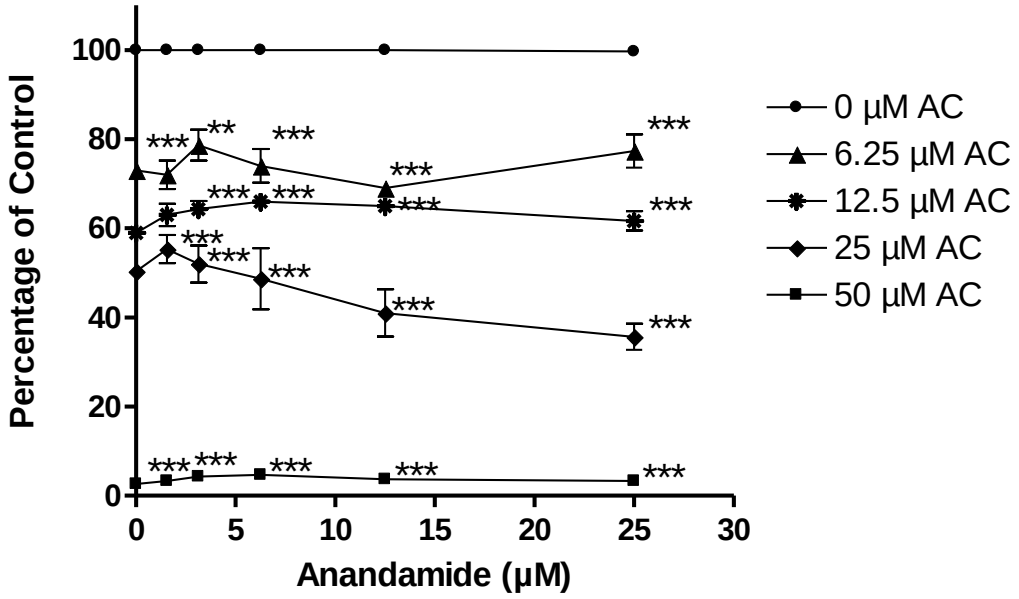


Figure 3.9. Percentage survival of stimulated lymphocyte cultures, after treatment with various concentrations of a combination of Rimonabant and anandamide. Results expressed as percentage survival of double treated cultures compared to the relevant untreated control values. *p < 0.01, **p < 0.001, ***p < 0.0001 compared to the untreated control

3.5 Discussion

The cytotoxicity of ethanol was tested to ensure that the measured cytotoxic effect of Rimonabant and anandamide, presented in the results, was undoubtedly due to the drug and not due to the effects of ethanol (Rimonabant and anandamide were dissolved in ethanol as described in 3.3.1). The results showed that ethanol had a cytotoxic effect on HUVEC at a concentration of 5% and higher. The concentration of ethanol present in the wells after addition of the drug concentrations used to treat the cells in this study was always much lower than 5%. The results thus allowed for the exclusion of ethanol as a source of cytotoxicity during these IC_{50} determinations.

The observed data illustrates that the cannabinoid receptor ligands mediated the inhibition of growth and survival of both HUVEC and lymphocytes. To investigate the effects of the investigational products on cell viability, HUVEC and lymphocytes were cultured in the presence of the synthetic cannabinoid Rimonabant and in the presence of

the endocannabinoid, anandamide. Both Rimonabant and anandamide had a dose related anti-proliferative response when tested on HUVEC. The same dose related response was observed during the Rimonabant exposure to lymphocytes, but no decrease in lymphocyte viability was observed when treated with anandamide at the concentrations tested (1.56 µM, 3.125 µM, 6.25 µM, 12.5 µM and 25 µM).

It is evident from the results that there was an almost ten times difference in the IC_{50} value of the two different products (14.3 µM for Rimonabant and 124.2 µM for anandamide) which was statistically significant. The results showed that exposure to Rimonabant at concentrations of 9 µM or higher, led to a significant decrease in the number of viable cells. This result was similar to that of McKallip *et al*., (2002) who tested THC (major psychoactive component of marijuana) on murine lymphomas and mastocytoma cells, as well as, human lymphoblastic leukaemia primary cell cultures. McKallip *et al*., (2002) indicated that these murine and human cell lines showed a significant reduction in cell viability when exposed to this cannabimimetic agent at concentrations of 10 µM or greater. A similar reduction in growth was observed during a study where human transformed mantle cell lymphoma cells were cultured in the presence of 10 µM of Rimonabant (Flygare *et al*., 2005). Anandamide on the other hand, produced a significant reduction in cell viability at concentrations of 75 µM and higher when tested on HUVEC. Nakajima *et al*. (2005) attempted to determine the cytotoxic effect of anandamide on human gingival fibroblasts, but found that these cells were unaffected by the endocannabinoid at concentrations of up to 20 µM. There is thus a significant difference in the cytotoxicity of the natural endogenous ligand, anandamide compared to the synthetic antagonist, Rimonabant. This difference might be ascribed to the metabolism of the different ligands. It is well known that anandamide is extremely unstable and quickly hydrolysed intracellularly by fatty acid amide hydrolase (FAAH) to produce ethanolamine and arachidonic acid (Svizenska *et al.*, 2008). During a study by De Petrocellis *et al*. (1998) it was determined that the cytotoxic effect of anandamide was potentiated with inhibition of FAAH. The exact metabolic process of Rimonabant however, is still unknown but would not involve FAAH. Rimonabant is probably not

metabolised as rapidly as anandamide resulting in longer exposure to the cells during culturing and increasing toxicity.

Studies have indicated that the anti-proliferative actions of anandamide are counteracted in the presence of Rimonabant with breast cancer cells (De Petrocellis *et al*., 1998) and prostate cancer cell lines (Sarfaraz *et al.*, 2005). Rimonabant combined with anandamide, at different concentrations, was tested on human lymphocytes to determine the possible synergistic effects on non-cancerous cells. No reduction in cytotoxicity was observed when the combined compounds were used at any of the concentrations tested. Instead an additive negative effect on viability was observed following the combination of 6.25 µM anandamide with 25 µM Rimonabant in the case of resting lymphocytes and the combination of anandamide 25 µM/ Rimonabant 25 µM and anandamide 12.5 µM/ Rimonabant 25 µM in stimulated lymphocytes. The cell survival at the higher doses of Rimonabant was very low and this could be an experimental artefact due to residual or background activity measured by the experimental technique. These results echo the result obtained during previous studies on transformed mantle cell lymphoma (Flygare *et al*., 2005). Flygare *et al.* (2005) observed a decrease in cytotoxicity to cancer cells during combination treatment of Rimonabant and anandamide, but showed an increase in toxicity when exposed to transformed cell lines. These synergistic anti-proliferative effects might be specific to primary cancer cells lines. The increased toxic synergistic results obtained during this experiment were at very high, non-physiological concentrations of Rimonabant and would not be of much use in further experiments.

Nakajima *et al.* (2006) obtained promising anti-inflammatory results using anandamide (1 µM, 5 µM and 10 µM) combined with CB_1 specific antagonists (AM251 or SR 144528) at a concentration of 1 µM.

3.6 Conclusion

The maximum concentrations at which Rimonabant and anandamide have insignificant cytotoxic effect on HUVEC and human lymphocytes were determined to find suitable concentrations for further experimentation with respect to the anti-inflammatory effects

of these compounds. The results obtained during this investigation succeeded in answering these study objectives and led to the following conclusions:

i. The cytotoxic effect on the cells treated with the drugs (Rimonabant and anandamide) can be ascribed to the drugs only and not to the ethanol used to dissolve these drugs.

ii. Rimonabant had an IC_{50} value of 14.3 µM when tested on HUVEC. The results indicated however, that Rimonabant initiated a cytotoxic effect from a concentration of 6.25 µM. To ensure minimal cytotoxicity during further experimentation, Rimonabant was used at concentrations lower than 6.25 µM. Anandamide demonstrated an IC_{50} value of 124.2 µM when tested on HUVEC. The results indicated that anandamide initiated a cytotoxic effect from a much lower concentration of 25 µM. These results provided a guideline as to what concentrations of anandamide could be used during further experiments. All further experiments were conducted using anandamide concentrations lower than 25 µM to ensure minimal cytotoxic effect on the HUVEC.

iii. Rimonabant and anandamide were tested on human lymphocytes and the cytotoxicity results were similar to those obtained during the experiments using the cultured HUVEC. Rimonabant had a cytotoxic effect on resting and stimulated human lymphocytes at a concentration of 10 µM and higher whereas anandamide had no cytotoxic effect at concentrations of up to 25 µM on lymphocytes.

iv. Synergistic effects were obtained at very high, non-physiological concentrations of Rimonabant during these experiments and it would not have been feasible to conduct experiments at such high concentrations.

The results collected during this phase of the study showed potential use of Rimonabant and anandamide at moderate and physiologically achievable concentrations and the lower concentration limits were used as an upper concentration limit during the rest of this investigation.

Chapter 4: Complement Receptor 3 (CR3) expression

4.1. Introduction

This chapter focuses on determining the *in vitro* effects of Rimonabant and anandamide on the expression of the CR3 receptor by neutrophils. CR3 expression is one of many crucial steps in the initiation of inflammation that could have anti-inflammatory effects if inhibited. Determining Rimonabant's effect on CR3 expression could include or exclude the possibility that Rimonabant's mechanism of action involves altering cell surface CR3 expression.

The complement system plays a key role in the host defence against invading micro-organisms by mediating destruction of these micro-organisms as well as initiating an inflammatory response (Tizard, 1992). Jules Bordet first discovered the complement system and described it as a heat-labile component present in normal plasma that enhances the opsonisation (a process by which a pathogen is marked for ingestion by a phagocyte) and killing of bacteria by antibodies (Figure 4.1) (Janeway *et al*., 2005). The complement system can be activated by the interaction between an antigen and an antibody and facilitates the effect of the antibody. It is clear that the complement system is essential to supplement the bactericidal effects of antibody dependant immunity (Nairn *et al*., 2002). Even though the complement system has been described as an effector of the antibody response, it can also be activated early in infection in the absence of antibodies (Janeway *et al*., 2005).

Many distinct plasma proteins make up the complement system and these sequentially react with one another in a cascade effect. These reactions opsonize pathogens and induce a series of inflammatory responses to help fight infection (Janeway *et al*., 2005). Although there is a large number of complement components, there are only nine basic complement proteins named C1-C9. Upon activation, several of the complement components are hydrolysed to form two functional fragments (Nairn *et al*., 2002). The smaller of the fragments is termed the 'a' fragment and the larger of the products is

termed the ‘b’ fragment (Weir *et al*., 1993). Each of the different fragments has different functions and can bind to different dedicated cell surface receptors.

Invading pathogens can activate the complement cascade through one or more of the following pathways: the classical pathway, the mannose-binding lectin pathway or the alternative pathway (Janeway *et al*., 2005). Activation of these pathways generates the complement fragments, which are able to amplify the inflammatory response by attracting and activating neutrophils, monocytes and lymphocytes to the site of infection (Stites *et al*., 1991). Male *et al*. (2006) summarise the functions of the complement system to include:

- The triggering and amplification of inflammatory reactions
- An important role in the development of antibody responses
- Cellular activation
- Attraction of phagocytes by chemotaxis
- Direct microbial killing and
- Clearance of immune complexes

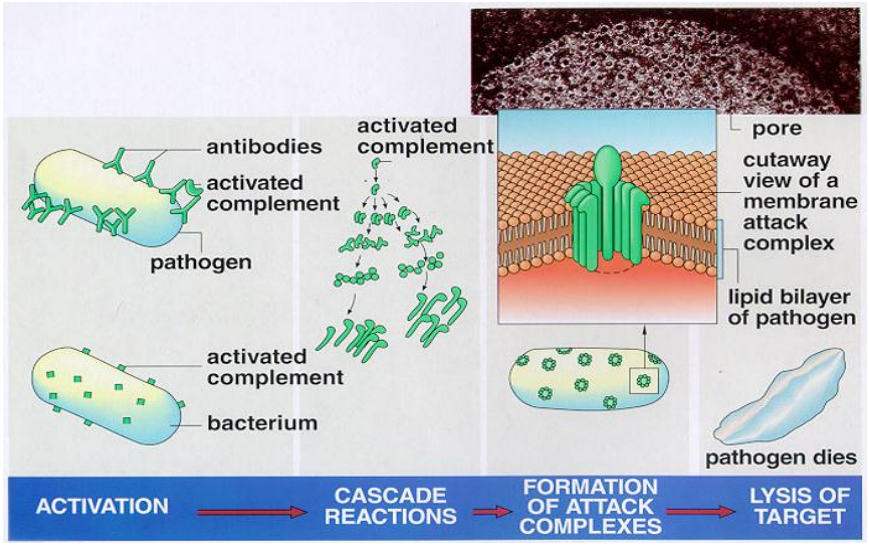


Figure 4.1. A diagrammatic representation of the activation of the complement system (Muller, 1998.).

The most important action of the complement system is to facilitate the ingestion and destruction of pathogens by phagocytic cells. This process is facilitated by complement receptors expressed on the surface of the phagocytes. These complement receptors recognise and bind to the complement components bound to the surface of the pathogens. CR1, CR2, CR3, CR4, CR5a and CR3a are the six major known complement receptors expressed in the body to which complement proteins bind. These receptors are expressed on different cell types and their functions vary. CR2 (CD21), CR3 (CD11b/CD18) and CR4 (CD11c/CD18) are the complement receptors that bind to the C3b complement fragment (the main effector molecule of the complement system), that attaches to pathogen surfaces (Janeway *et al*., 2005).

CR3 binds specifically to the C3 fragment called iC3b. CR3 together with lymphocyte functional antigen-1 (LFA-1) and CR4 are members of the integrin receptor family. CR3 differs from the other complement receptors due to the fact that it binds to iC3b in a calcium dependent manner. CR3 is expressed on the surface of neutrophils, macrophages and NK cells (Tizard, 1992). CR3 plays a major role in cell adherence, aids in ingestion, facilitates diapedesis of cells to extravascular sites and acts as co-factor for further degradation of C3bi (Stites *et al*., 1991).

Neutrophils are not normally found in tissues unless recruited from the circulation to sites of tissue inflammation (Stites *et al*., 1991). Once in the peripheral tissues, the neutrophils carry out their function of phagocytosis (Turgeon, 1996). Neutrophils are short-lived cells that phagocytise invading or damaged material, destroy it and die off in the process (Nairn *et al*., 2002).

Individuals who lack particular components of the complement system highlight the importance of this system in the immune defence of the body, for example, children who lack the component C3 are subject to devastating repeated bacterial infections. However, although required for normal physiological function, most elements of the immune system, including the complement system can cause harm to the host if it is over activated or activated inappropriately (Male *et al*., 2006). The complement system is

known to be involved in the pathology of numerous diseases, opening an opportunity to develop therapies that can control the complement activation. The key role of compliment receptors in the initiation of the inflammatory responses of the phagocytic cells provides motivation for examining Rimonabant's possible inhibitory action on the expression of CR3 by neutrophils.

4.2. Aim

The purpose of this study was to determine the *in vitro* effects of Rimonabant and anandamide on the surface expression of the CR3 complement receptor and adhesion molecule by human neutrophils.

4.3. Materials and methods

The method used during this experiment to determine CR3 expression by neutrophils had previously been used and described by scientists including Graham *et al.* (1989) and Nicholson *et al*. (2007). This method was repeated 5 times with 5 different blood donors for each drug tested, i.e. Rimonabant and anandamide. Rimonabant and anandamide were tested in separate independent experiments at different drug concentrations. Rimonabant was tested at 3.12 µM, 6.25 µM, 12.5 µM, 25 µM and 50 µM whereas anandamide was tested at 12.5 µM, 25 µM, 50 µM, 100 µM and 200 µM. These concentrations were selected according to the cytotoxicity of the individual compounds described in the results of Chapter 3. The isotypic control was incorporated into the experiment to account for non-specific binding by the type of antibody used. The isotypic control uses a mouse immunoglobulin G (IgG) antibody to verify that the CD11b antibody binds specifically to CR3.

The patients used in this study were selected according to certain inclusion and exclusion criteria to ensure healthy neutrophils for this experiment. Each patient was required to sign an informed consent form confirming their authorization for the use of their blood during this research project (refer to Annexure C).

4.3.1. Preparation of reagents

The reagents were sterilised before use and stored in a sterile sealed containers. The containers were only opened inside a sterilised laminar flow cabinet (Labotec, model LCA 6000VA) to prevent contamination. Ammonium chloride and $RPMI^+$ were prepared according to the method described in Section 3.3.2. Rimonabant and anandamide were prepared according to the method described in Section 3.3.1.

i. Phorbol myristate acetate (PMA)

PMA (Sigma Aldrich, Johannesburg, RSA) stock solution was prepared by dissolving 1 mg PMA in 1 ml ethanol and stored at -70^oC (stock solution was closed under sterile conditions before storage). Twenty microliters of the PMA stock solution was added to 1 ml $RPMI^+$ medium just prior to use. This solution was diluted further by adding 100 µl of the new solution (20 µl PMA/ 1 ml $RPMI^+$) to 1.9 ml $RPMI^+$ medium. A 1/10 dilution into the test solution provided a final concentration of 100 ng/ml PMA.

ii. Monoclonal antibody

A PE-conjugated antibody targeting CD11b- (a phycoerythin conjugated IgG from mouse targeting the CD11b portion of the CR3 receptor) purchased from Beckman Coulter (PN IM258IU) was used in this experiment to give a relative quantitation of surface expressed CR3 receptors on human neutrophils after exposure to either Rimonabant or anandamide. Non-specific binding was compensated for by inclusion of a relevant isotypic control. Mouse IgG_1-FITC purchased from Beckman Coulter, was the antibody used for the isotypic control.

iii. Isoflow

Isoflow^{TM} EPICS^{TM} Sheath fluid was used undiluted and supplied by Beckman Coulter, Halfway House, RSA.

4.3.2. Experimental procedures

Venous blood was collected by venepuncture of a healthy volunteer into ethylendiaminetetraacetic acid (EDTA) anticoagulant tubes. Sterile centrifuge tubes were

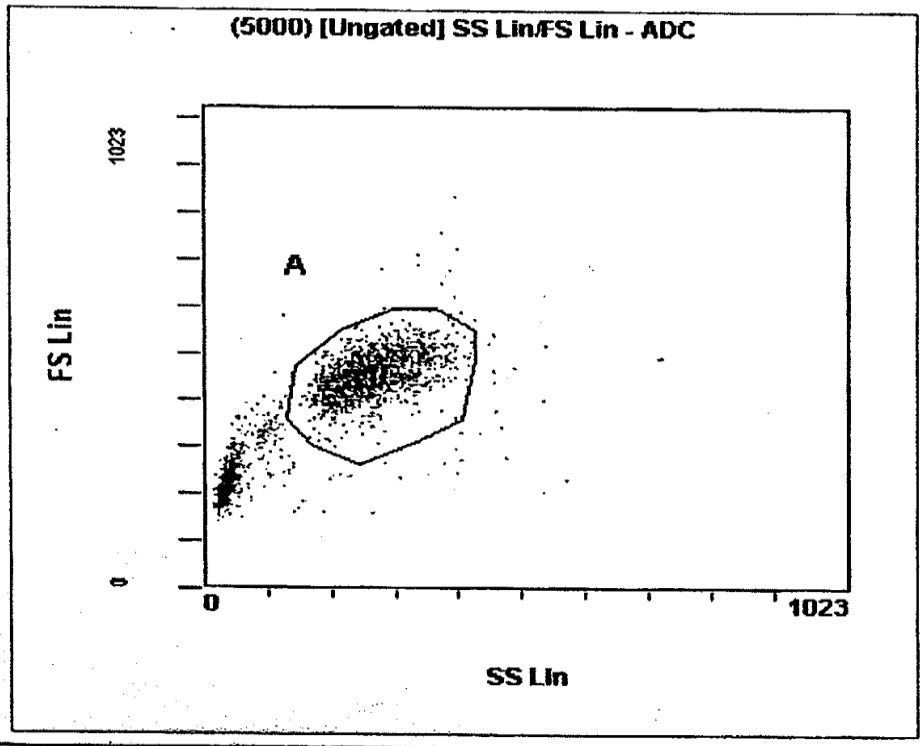
labelled as follows: Resting isotypic control (1 tube), Stimulated isotypic control (1 tube), Resting control (1 tube), Stimulated control (1 tube), Resting (5 different compound concentrations each in a separate tube), Stimulated (5 different compound concentrations each in a separate tube). The EDTA blood (500 µl) was placed into each of the 14 tubes. All 14 tubes (7 ml tubes) were then filled with ice cold ammonium chloride solution and allowed to stand on ice for 10 minutes (ammonium chloride is used to lyse the red blood cells). The tubes and their contents were centrifuged for 10 minutes at 200 *g*. This was done to isolate the leukocytes. The supernatant (ammonium chloride and lysed cells) was removed and the pellet (mixed lymphocytes) was resuspended in $RPMI^+$ medium. The tubes and their contents were once again centrifuged for 10 minutes at 200 *g*. The last step was repeated twice (this was done to wash the cells and to ensure complete absence of ammonium chloride). $RPMI^+$ medium (300 µl) was added to each of the 14 tubes and the cell suspension was mixed repeatedly by pipetting with an automatic pipette to ensure a uniform suspension. The tubes containing the cell suspension were incubated in a 37^oC water bath for 15 minutes to allow the cells to re-equilibrate. Fifty microliters of the various concentrations of the test compounds (Rimonabant/anandamide) was added to the specific tubes and 100 µl $RPMI^+$ medium was added to the controls. The cell suspension was incubated in a 37^oC water bath for a further 15 minutes. One hundred microliters of PMA was added to the appropriate tubes to stimulate the cells. The resting cells received only 100 µl of $RPMI^+$ medium to the tubes. The cell suspension was allowed to incubate in a 37^oC water bath for a further 30 minutes. Five microliters of the monoclonal antibody (anti-CD11b-PE) was dispensed into the corresponding flow cytometer sample acquisition tubes (labelled to correspond to centrifuge tubes). The corresponding cell suspension (100 µl) was added to the antibody containing tubes and incubated for 10 minutes in the dark followed by the addition of 400 µl of Isoflow to the cell-antibody mix. This final mixture was analysed on a flow cytometer (Beckman Coulter FC500). The cell population corresponding to the neutrophils in the scattergram were gated and analysed for mean fluorescent activity of fluorescent channel 2 of the entire neutrophil population (Figure 4.2). The results were presented in graphical format as shown in Figure 4.3 and Figure 4.4.

4.4. Results

The mean, SEM and the graphs were obtained by using the GraphPad Prism 4 software package. Each value represents the mean of 5 independent experiments. The 5 repetitions of this experiment had similar results. Both resting and PMA stimulated neutrophils were tested. The level of significance of the differences between the means of fluorescence values or ratios of different samples was determined with Kruskal-Wallis test.

As shown in Figure 4.3 and Figure 4.4, there is a large increase in fluorescence of the cells stimulated with PMA as compared with the resting cells, and this was expected due to treatment with a known inflammatory stimulant. There was however, almost no distinction in the florescent intensity emitted by the cells treated with the different concentrations of the test compounds used in this experiment compared to the untreated control (Figure 4.3 and Figure 4.4). As illustrated in Figure 4.3, the fluorescent intensity of the resting neutrophils treated with Rimonabant, appeared to decrease slightly in a dose dependant manner compared to the untreated control groups. Rimonabant thus appears to have a slight inhibitory effect on the CR3 expression of resting neutrophils at concentration of 6.25 µM and higher, however these differences were not statistically significant.

Figure 4.4 illustrates that anandamide treatment appeared to show a slight increasing dose response on the expression of CR3 when comparing PMA treated neutrophils but showed no statistical significance. No effect could be observed for resting neutrophils.



(5000) [Ungated] SS Lin/FS Lin

Region	Number	%Total	%Gated	X-Mean	X-CV	Y-Mean
ALL	5000	100.00	100.00	184	69.64	370
A	3008	60.16	60.16	264	20.19	455

Figure 4.2. A typical flow cytometric scattergram of a mixed leucocyte population showing the forward scatter (FS) and side scatter (SS) pattern of the different cells. The lymphocyte population is close to the Y axis with the neutrophil population that has been “gated” for further analysis of fluorescence by these cells only. The smaller monocyte population lies between these two cell groupings. Fluorescence intensity analysis of the gated neutrophils was used to quantitate the expression of CR3 following staining with PE fluorescently tagged anti-CD11b monoclonal antibodies.

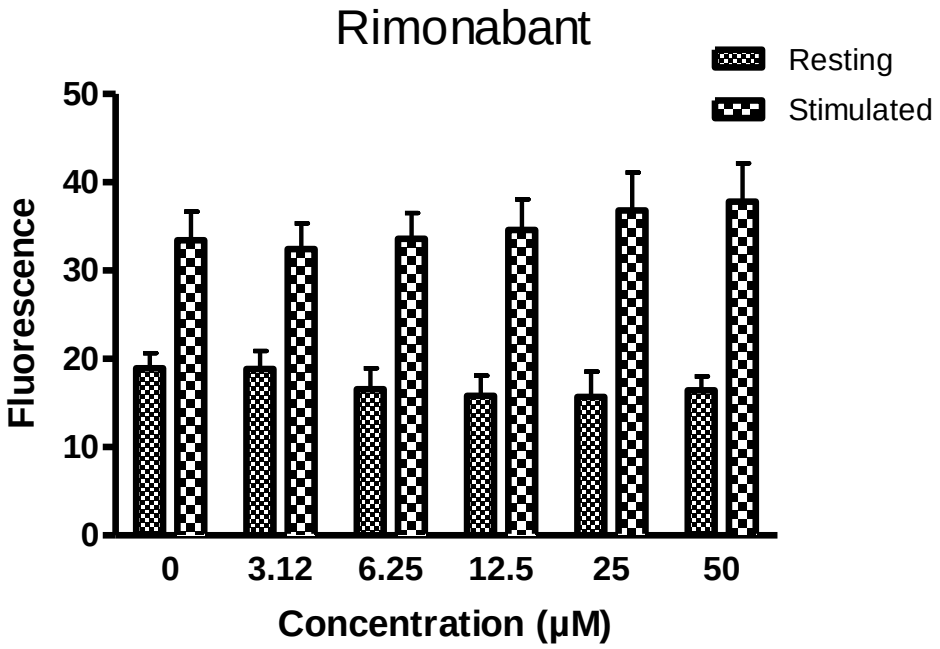


Figure 4.3. The effect of various concentrations of Rimonabant on the CR3 expression by human neutrophils.

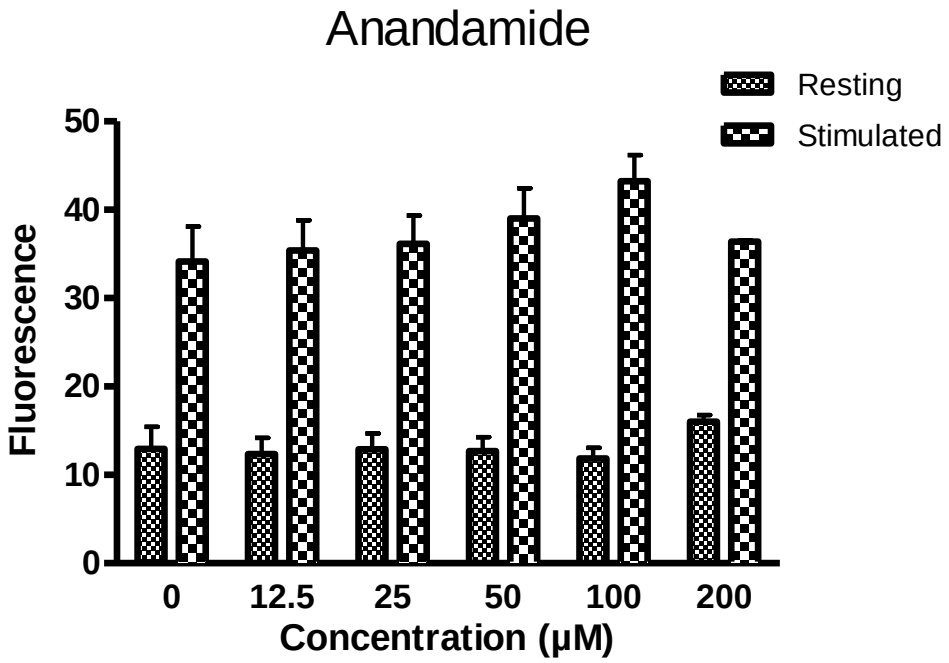


Figure 4.4. The effect of various concentrations of anandamide on the CR3 expression of human neutrophils.

4.5. Discussion

In this study a direct immunofluorescence staining method coupled to flow cytometric analysis was used to determine the relative quantity of CR3 on the cell surface of neutrophils exposed to different concentrations of the test compounds. This was achieved by running samples containing a fluorescently labelled antibody bound to neutrophils through the flow cytometer. The flow cytometer is designed to deliver the cells in single file at the point of measurement in the flow cell. The sample is injected into the centre of a stream of liquid that is narrowed to a diameter of approximately 20 µm. This stream constrains the cells to move through the centre of the flow cytometer chamber, at which point a beam of laser light is focused onto the hydrodynamically focused stream of fluid. A number of detectors are aimed at the point where the stream passes through the light beam to detect the forward scatter (light scatter in line with the light beam), the side scatter (light scatter perpendicular to the light beam) and the specific fluorescence intensity emitted from the individual cells. The direct staining method used has the benefit of requiring only one antibody incubation step and eliminates the possibility of non-specific binding from a secondary antibody (Ormerod, 2008).

Human neutrophils were prepared, treated with various concentrations of Rimonabant and anandamide separately and then reacted with the PE-conjugated monoclonal antibody to the ligand binding site of CR3 as previously described by Nicholson *et al.* (2007). A CD11b-PE monoclonal antibody was used during this experiment.

An appropriate isotype control antibody was selected for this flow cytometry experiment to distinguish between the non-specific (background) staining and the specific antibody binding needed to determine the extent of CR3 expression. The isotypic control needs to be matched to the specific primary antibody (species and isotype) being used in order to accurately determine the level of specific staining by the primary antibody. For this reason untargeted mouse IgG_1-FITC was used as an isotypic control for the CD11b-PE monoclonal antibody used during this study.

CR3 functions not only as a receptor for phagocytosis and cytotoxicity, but also as an adhesion molecule responsible for leukocyte diapedesis. Therefore CR3 plays a vital role in the initial inflammatory response. When CR3 expression by immune cells is decreased, a decrease in inflammatory response and extravacularisation is expected. The role of CR3 during the initiation of an inflammatory response formed the rational for this experiment to determine the possible inhibitory effects of Rimonabant and anandamide on CR3 expression.

In the presence of these concentrations, neutrophils exhibited little if any decrease in fluorescence intensity and showed no dose response at all for either of the test compounds.

This result indicates that neither Rimonabant nor anandamide significantly affect CR3 expression on the surface of freshly isolated human neutrophils and would exclude the CR3 expression pathways as a potential mechanism of action for the anti-inflammatory effects of these compounds.

The unusual low levels of CR3 expressed during this experiment when compared to other research articles, is however worth exploring. Shalekoff *et al*. (1998) reported that EDTA used as an anticoagulant during the preparation of the whole blood, significantly increased the expression of CD11b on PMN. According to Shalekoff *et al.* (1998) the choice of anticoagulant as well as an increase in temperature had a marked influence on activation marker expression. This publication suggests that when testing the expression of CD11b, heparin would be the better choice to use as an anticoagulant. This might be because IL-8 is produced in the presence of heparin at levels approximately 20-fold higher than those expressed in the presence of EDTA and a major effect of IL-8 is the up-regulation of CD11b on PMN (Shalekoff *et al.,* 1998). Perhaps heparin would produce better expression of CD11b, although the Shalekoff *et al.* (1998) results are based on an 18 hour incubation period at room temperature and 37^{o}C to produce the inhibitory effects observed. During the current experiment, the whole blood was stored at room temperature for no longer than 15 minutes before it was placed on ice. The effect on the

expression of CD11b mentioned in the Shalekoff *et al.* (1998) publication was greater at 37^{o}C and might not be significant in the current method used. This hypothesis would need to be tested by repeating this experiment using heparin instead of EDTA to evaluate the CR3 expression.

On the other hand, Rimonabant might possibly exert its anti-inflammatory properties by inhibiting other neutrophil macromolecule expression such as neutrophil elastases.

4.6. Conclusion

Rimonabant and anandamide had no significant effect (stimulatory or inhibitory) on the expression of CR3 by resting or PMA stimulated human neutrophils at concentrations that would be physiologically achievable. The results of this experiment eliminated the possibility that these cannabinoid ligands exert anti-inflammatory effects by inhibiting the CR3 expression of human neutrophils; however confirmation is needed to eliminate the possibility of inhibitory effects of the anticoagulant used during this experiment.

A different anti-inflammatory mechanism of action would however be followed as sufficient evidence of an anti-inflammatory effect has been reported. These alternative mechanisms of action could possibly be the inhibition of the production of cytokines or the inhibition of ICAM-1 expression by the endothelial cells of the vasculature. Further investigation and experimentation was essential to prove these hypotheses.

Chapter 5: Cytokine production by human macrophages and HUVEC

5.1. Introduction

Cytokines are essential protein based mediators of the body's immune response and can trigger or shutdown inflammation. By assessing the influence that Rimonabant has on the release of various cytokines, an indication of whether cytokine control could be the mechanism of action can be determined, which could be of therapeutic interest.

Cells that mount an innate immune response against micro-organisms such as bacteria, viruses and fungi that penetrate the epithelial surfaces of the body do so immediately after invasion. Phagocytic cells exert the defence against these organisms by recognising them as foreign, engulfing them and destroying them by one of several mechanisms. The recognition of organisms as foreign is due to the presence of surface receptors on the phagocytic cells. Binding of the phagocytes to the organism's cell wall triggers the phagocytes to engulf the foreign organism (Figure 1.2) and induces phagocytes to secrete biologically active messenger molecules including cytokines. Cytokines are small proteins released by various immune cells in the body and can be described as messenger proteins or mediators. Cytokines released by activated cells affect the behaviour of other cells that bear receptors for that specific cytokine (Figure 5.1) (Janeway *et al*., 2005). Cytokines act as humoral regulators at nano- to picomolar plasma concentrations either under normal or pathological conditions (Goncharova *et al*., 2007). The cytokines released by activated phagocytes in response to bacterial, viral or fungal pathogens, initiate the complex process known as inflammation (Janeway *et al*., 2005).

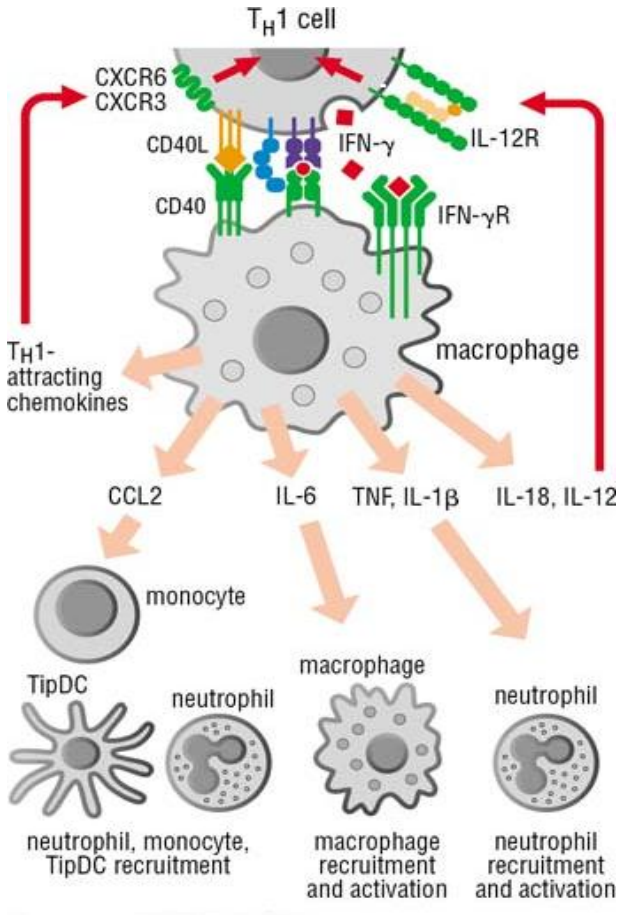


Figure 5.1. A diagrammatic representation of the cells affected by the release of cytokines (www.new-science-press.com).

Cytokines can exert their effect in one of the following ways: by acting in an autocrine manner (affecting the behaviour of the cells that releases the cytokine), by acting in a paracrine manner (affecting the behaviour of adjacent cells) or by acting in an endocrine manner (affecting the behaviour of distant cells). Cytokines modulate the reactions of the host to foreign organisms by regulating the growth, mobility and differentiation of leukocytes and other cells involved in the immune response (Stites *et al*., 1991).

Approximately 100 different human cytokines have been discovered to date (Goncharova *et al*., 2007). IL-1 is a pro-inflammatory cytokine produced by various cell types, including macrophages, keratinocytes, dendritic cells, astrocytes, microglial cells, normal B lymphocytes, cultured T cell clones, fibroblasts, neutrophils, endothelial cells and smooth muscle cells. IL-1 production by these cells are stimulated by a variety of agents,

including lipopopolisaccharide (LPS), muramyl dipeptide (MDP), injurious ultraviolet irradiation, phobol myristate acetate (PMA), aluminium hydroxide and micro-organisms (Stites *et al*., 1991). IL-1 exists in two molecular forms that are encoded by different genes. The two forms are called IL-1α and IL-1β, both of which the potency and activities are virtually identical. The blockade of IL-1 has showed remarkable efficacy in diseases such as cryopyrinopathies, gout, still syndrome and IL-1Ra deficiency. It has been proven that a specific antagonist against IL-1 production is of therapeutic interest, due to IL-1 involvement in chronic inflammatory diseases (Rothe *et al*., 2011).

Tumour necrosis factor (TNF) is one of the major pro-inflammatory cytokines produced by cells of the innate and adaptive immune system, which contributes to the pathogenesis of various diseases (Rothe *et al*., 2011). TNF is produced by a variety of cells, including macrophages. TNF can be produced by a large variety of cells stimulated by various agents including: LPS, PMA, MDP and foreign antigens (Stites *et al*., 1991). The vascular endothelium is a major site of action for TNF-α. TNF-α induces inflammatory responses of the vascular endothelium by enhancing the surface expression of adhesion molecules and the secretion of inflammatory other mediators (Nizamutdinova *et al*., 2007). TNF and IL-1 are major "broad-spectrum" inflammatory mediators and have largely the same immunologic activities. TNF inhibitors form a segment of the therapy of various rheumatic diseases by neutralizing soluble and membrane-bound TNF. There are currently five different biological TNF inhibitors available for clinical use (Rothe *et al*., 2011).

IL-6 is a cytokine with multiple biologic activities that mediates its effects by binding to a soluble or membrane bound IL-6 receptor. IL-6 has been characterized as a pleiotropic driver in acute and chronic inflammation (Rothe *et al*., 2011). IL-6 has an effect on a variety of cells, including B cells, myelomas, hepatocytes and T cells. IL-6 can be produced by many cells, including T and B lymphocytes, monocytes, endothelial cells, epithelial cells and fibroblasts. A variety of stimuli including TNF, IL-1, antigens, mitogens and LPS induce the production of IL-6 (Stites *et al*., 1991). IL-6 has been shown to be involved in joint inflammation, but is also essential for systemic features of

inflammation such as fever, fatigue, anaemia and the increase in plasma acute-phase proteins (Rothe *et al*., 2011).

IL-8 is primarily produced by monocytes and induces chemotaxis and activation of neutrophils once released in the body. IL-8 is also known to induce degranulation of lysosomal enzymes and induces the expression of adhesion molecules (Miyamoto *et al*., 2002). Studies indicate that mice lacking IL-8 receptors are less susceptible to atherosclerosis and have fewer monocytes accumulated in vascular lesions (Yi-Yuan *et al*., 2004).

T lymphocytes, B lymphocytes and macrophages produce IL-10 whereas IL-12 is thought to be produced by T lymphocytes only. IL-12 activates NK-cells during an inflammatory response (Roitt, 1994). Recent studies have confirmed the significance of IL-12 in the immune-pathogenesis of psoriasis. Two compounds (ustekinumab and ABT-874) directed against IL-12, were studied and resulted in significant improvement of cutaneous lesions in psoriasis patients (Rothe *et al*., 2011).

Some transformed lymphocyte, macrophage, keratinocyte and fibroblast cell lines spontaneously secrete cytokines into the culture medium. This spontaneous release is an exception to the rule, which normally requires cells to be stimulated in order to produce cytokines (Stites *et al*., 1991).

Macrophages play a major role in the early defence of the body against foreign invading antigens and are involved in both natural and acquired immunity. Macrophages have many functions that promote inflammation and even wound healing. Macrophages contribute to these events through phagocytosis and antigen presentation as well as by producing and secreting biologically active factors including enzymes, eicosanoid products and cytokines (Klein *et al*., 1998).

Cannabidiol (CBD) is a non-psychoactive compound found abundantly in marijuana. Experiments done by Tong-Rong *et al.* (2007) on BALB/c mice indicated that CBD

reduced the ability of splenic T cells to proliferate and to produce IL-2, IL-4 and IFN-γ. These results aided in the confirmation of their hypothesis that CBD had a marked immunosuppressive effect on ovalbumin-sensitised mice. A study done by Srivastava *et al.* (1998) reported the reduction of IL-10 production by HUT-78 T cells treated with CBD.

((-)-Δ^9-tetrahydrocannabinol (THC) acts as an agonist for cannabinoid receptors in the body. A recent study confirmed the theory that THC inhibits the inflammatory response by testing Legionella infected dendritic cell cultures. Dendritic cells infected with Legionella produced a high level of IL-12p40, which was significantly inhibited when treated with THC in a concentration dependent manner (Lu *et al*., 2006). It has been reported that THC also inhibits the production of IL-8, TNF-α, IFN-γ and IL-10 by various cell types (Srivastava *et al*., 1998)

1', 1' –dimethylheptyl-THC-11-oic acid (trivial name, ajulemic acid {AjA}) is one of the compounds found in the class of cannabinoids called the THC acids. AjA is free from cannabimimetic central nervous system activity, but suppressed the production of IL-1β during an experiment done *in vitro* on human mononuclear cells (Zurier *et al*., 2003). This experiment indicated that some THC acid derivatives have anti-inflammatory properties without having any psychoactive effect on the human brain.

In a study done by Smith *et al.* (2001), the anti-inflammatory properties of CB_1 receptor agonists were confirmed. [L-(-)-11-hydroxy-Δ^8 tetrahydrocannabinol-dimethylheptyl] (HU-210) and {(R)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl[pyrrolo[1,2,3-de]1,4- benzoxazin-6-yl] (1-naphthalenyl methanone} (WIN 55212-2) both inhibited the production of the inflammatory cytokines TNF-α and IL-12 and were found to bind to CB_1 receptors.

A study done by Gallily *et al.* in 2000 reported that the endocannabinoid, 2-AG inhibited the production of TNF-α by murine peritoneal macrophages (*in vitro*) as well as in BALB/c mice. The *in vitro* results indicated a dose response and reported a 90%

inhibition of TNF-α production after a 24 hour treatment period with 2-AG at a concentration of 10 µg/ml (Gallily *et al*., 2000).

The concentrations and combinations of test compounds used in the study reported here are similar to those used by Nakajima *et al.* (2006) who tested anandamide and AM251 (a selective CB_1 antagonist) on human gingival fibroblasts for the effects on extra cellular IL-6, IL-8 and MCP-1 levels using an ELISA assay. An ELISA assay is a quantitative immunologically based technique used to quantitate a particular protein or molecule in a sample. Anandamide significantly reduced the production of all the tested cytokines and this inhibitory effect was attenuated by AM251.

Miyamoto *et al*. (2002) reported that HUVEC, stimulated with bleomycin, expressed IL-8 and monocyte chemo-attractant protein-1 (MCP-1). This was also determined by using an ELISA assay for these cytokines.

Given the importance of cytokines, particularity IL-1 and TNF-α in an inflammatory response and the ability of cannabinoids (as seen in previous studies) to inhibit the production of these cytokines, a possible mechanism of action for Rimonabant in relieving the symptoms of chronic inflammation could be by inhibiting cytokine production.

The objective of this chapter was therefore to address the question: Does Rimonabant exert an effect on the production of cytokines by human macrophages and HUVEC? Answering this question can lead to a better understanding of the mechanism of action of AC and its possible anti-inflammatory properties.

5.2. Aim

The aim of this study was to determine the *in vitro* effect of Rimonabant (AC) and anandamide (AEA) alone and in combination on the production of cytokines by human macrophages and by HUVEC.

5.3. Materials and Methods

The methods used to determine the cytokine production was repeated 8 times for each cell type (human macrophages and HUVEC). Eight different blood donors for macrophage isolation and eight different umbilical cords for HUVEC isolation were used during this experiment. Eleven different drug combinations, using Rimonabant at 0.5 µM, 1 µM, 3 µM and anandamide at 5 µM, 10 µM, as well as two control groups were used in this investigation as described below in Figure 5.2 and Figure 5.3. These combinations of concentrations are similar to the concentrations used by Nakajima *et al*. (2005).

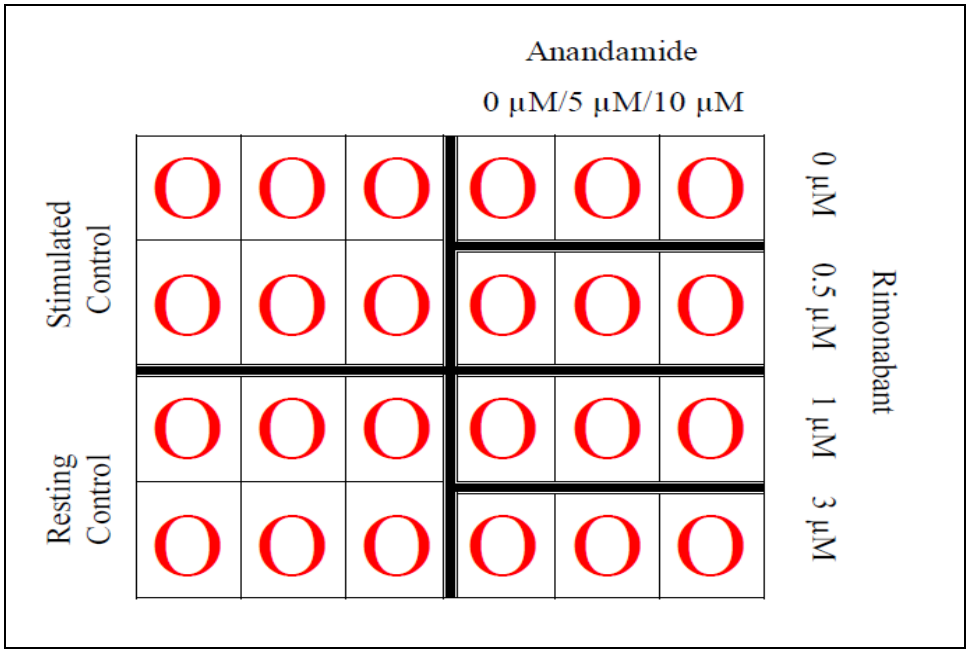


Figure 5.2. A diagrammatic representation of the different drug concentrations and combinations used to determine the cytokine production by LPS stimulated macrophages. Three different concentrations of anandamide and four different concentrations of Rimonabant were tested in eleven different combinations in a 24 well tissue culture plate. Three tissue culture plates were used to attain all eleven combinations.

The patients used in this study were selected according to the inclusion and exclusion criteria to ensure healthy macrophages for the experiment. Each patient was required to sign an informed consent form verifying their authorization for the use of their blood during this research project (refer to Annexure C).

5.3.1. Preparation of reagents

The reagents were prepared, sterilised and stored in sterile airtight containers. The containers were only opened inside a sterilised laminar flow cabinet (Labotec, model LCA 6000VA) to prevent contamination of the reagents. Gelatine, trypsin and 199 medium+ were prepared according to the method described in Section 2.3.1. $RPMI^{+}$, ammonium chloride, Histopaque and cell counting fluid were prepared according to the method described in Section 3.3.2. Rimonabant and anandamide were prepared according to the method described in Section 3.3.1.

i. Bacterial lipopolysaccharide (LPS)

Lipopolysaccharide B from *E. Coli* was obtained from Difco Laboratories (Detroit, Michigan, USA). LPS (25 µg) was dissolved in 1 ml of the appropriate tissue culture medium. The final concentration of LPS used to stimulate the cells was 2.5 µg/ml.

5.3.2. Experimental procedures

i. Human macrophage preparation

All procedures were carried out under sterile conditions. Fresh blood was collected from the cubital vein of a healthy volunteer and heparinised (5 units heparin/ml blood). Blood (35 ml) was carefully loaded onto 15 ml Histopaque with a Pasteur pipette and centrifuged for 25 minutes at 650 *g* (at room temperature). The top plasma layer was placed in a sterile tube and used to supplement the medium (donor plasma) as described below. The lymphocyte-monocytes layer (± 12 ml) was transferred to a sterile 50 ml centrifuge tube and filled with sterile RPMI-1640 medium. The lymphocyte-monocyte cell solution was centrifuged for 15 minutes at 200 *g* to remove contaminating platelets and the residual Histopaque. The supernatant fluid was discarded and the pellet was resuspended in 50 ml sterile, cold ammonium chloride and left to stand on ice for approximately 10 minutes. This step was done to lyse any contaminating red blood cells present in the cell suspension. The cell suspension was centrifuged for 10 minutes at 200 *g*, the supernatant was discarded and the pellet was resuspended in $RPMI^{+}$ (the tube was filled with $RPMI^{+}$). The suspension was centrifuged once again for 10 minutes at 200 g

and the supernatant discarded. The cell pellet was resuspended in 5 ml RPMI (RPMI was supplemented with 5% donor plasma). The cells were counted (as described in Section 3.3.3.iii) with cell counting fluid and diluted in RPMI (supplemented with 5% donor plasma) to 1 x 10^6 cells/ml RPMI. The new cell suspension (2 ml) was placed into each well of a sterile 24 well tissue culture plate and incubated for 2 hours. A 24 well tissue culture plate was used to allow cells to incubate on a larger surface area and an increased volume of culture medium (2 ml compared to the 200 µl of a 96 well plate). After the incubation period, the tissue culture plate was removed from the incubator and rinsed 4 times with RPMI (supplemented with 5% donor plasma). This was done to remove all the non-adherent lymphocytes from the wells (monocytes attach to the bottom of the tissue culture plate). RPMI (2 ml supplemented with 5% donor plasma) was added to each well and the culture plate incubated in a 37°C/5% CO_2 incubator for 14 days. During this incubation period, the medium (RPMI supplemented with 5% donor plasma) was changed daily. Monocytes naturally release growth hormones into the medium, which is essential for the monocytes' transformation into macrophages. Therefore only half of the medium was removed daily from the wells and replaced with fresh medium to ensure monocytes' contact with these naturally released growth hormones.

ii. Preparation of HUVEC

HUVEC cell were harvested from 75 cm^2 culture flasks by trypsinisation and the pellet resuspended in 1 ml 199 medium+. The cell suspension was mixed thoroughly by pipetting repeatedly with an automatic pipette to make sure that the cells were separated from each other and that a uniform suspension was formed. The cells were counted using cell counting fluid (as described in Section 3.3.3.iii) and diluted in 199 medium+ to a concentration of 1 x 10^6 cells/ml 199 medium+. Sterile 96 well tissue culture plates were pre-coated with 1% gelatine solution (as described in Section 2.3.2.ii) before plating HUVEC into the wells. Equal amounts (100 µl) of the cell suspension and 199 medium+ were added to each of the gelatine coated 96 wells of the tissue culture plate. The HUVEC was incubated in a 37°C/5% CO_2 incubator for 72 hours before proceeding to the next step of the experiment. This incubation period allows time for the repair of any damage caused to the cell surface during trypsinisation.

iii. Cell proliferation assay of human macrophages

Three 24 well tissue culture plates containing human macrophages were divided into sections representing the different drug concentrations, as well as the control groups (Figure 5.2). All the medium was removed from the wells and 250 µl $RPMI^+$ was dispensed into each of the 24 wells and allowed to incubate for 24 hours in a 37°C/5% CO_2 incubator. After the incubation period, 20 µl of each experimental drug (various concentrations) was dispensed into the labelled wells and 40 µl of the $RPMI^+$ to the untreated control wells (Figure 5.2). The tissue culture plate was placed into a sterile container along with a small amount of water. This container was placed into a 37°C/5% CO_2 incubator and allowed to incubate for 48 hours. Following the incubation period, 20 µl of LPS was added to the stimulated wells and 20 µl of $RPMI^+$ to the resting wells. The tissue culture plate was placed back into the container and incubated for a further 24 hours. After the incubation period the medium in each well was removed, placed into corresponding labelled sterile micro-centrifuge tubes and stored at -70^oC until analysis took place.

iv. Cell proliferation assay of HUVEC

The 96 well tissue culture plates containing the HUVEC were divided into sections representing the different drug concentrations, as well as the control groups (Figure 5.3). All the medium was removed from the wells using a sterile Pasteur pipette. Fresh 199 medium+ (140 µl) was dispensed into each of the 96 wells and the plates were allowed to incubate for 24 hours in a 37°C/5% CO_2 incubator. After the incubation period, 20 µl of each experimental drug (various concentrations) was dispensed into the labelled wells with the addition of 40 µl of the 199 medium+ into the untreated control wells (Figure 5.3). The tissue culture plates were placed into a sterile container along with a small amount of distilled water. This container was placed into a 37°C/5% CO_2 incubator and allowed to incubate for 48 hours. Following the incubation period, 20 µl of LPS was added to the stimulated wells and 20 µl of 199 medium+ to the resting wells of the culture plates. The tissue culture plates were placed back into the container and incubated for a further 24 hours. After the incubation period the medium in each well

was removed, placed into corresponding labelled sterile micro-centrifuge tubes and stored at –70^{o}C until analysis took place.

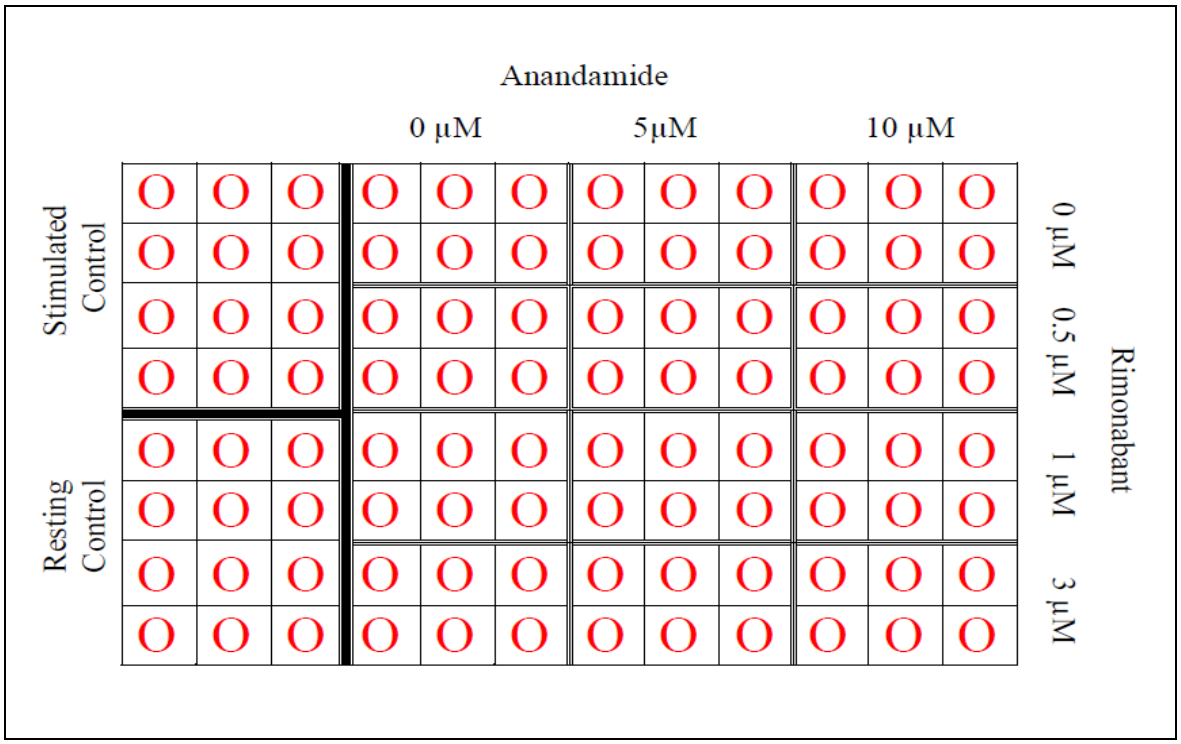


Figure 5.3. A diagrammatic representation of the different drug concentrations and combinations used to determine the cytokine production by LPS stimulated HUVEC. Three different concentrations of anandamide and four different concentrations of Rimonabant were placed in eleven different combinations in a 96 well tissue culture plate along with 2 different control groups.

v. Analysis of cytokine production

In this study the cytokine levels of the supernatant media from the test compound exposed macrophages or HUVEC were measured using a BD FACS Array™ flow cytometer using a BD Cytometric Bead Array Ready-to-use human inflammation kit. The kit could quantitate IL-8, IL-1, IL-6, IL-10, TNF-α and IL-12p70. A PE positive detector control was used with the cytometer setup beads to set the initial instrument compensation setting as per the manufacturers protocols (www.bdbiosciences.com).

This kit was selected due to its potential to simultaneously quantitate 6 soluble extracellular cytokines. The BD Cytometric Bead Array (BD CBA) assay is designed to use the selectivity of fluorescence using a flow cytometer where selectively immune-captured cytokines on identifiable particles are measured. The kit uses six different intensity red coloured capture beads each of which binds only a specific cytokine by fixed capture antibodies. The specific 'capture beads' have separate fluorescence intensity characteristics and they are distinguished from brightest to dimmest as follows:

	Bead	Specificity	
Brightest	A1	IL-8	
	A2	IL-1β	
	A3	IL-6	
	A4	IL-10	
	A5	TNF	
Dimmest	A6	IL-12p70	(www.bdbiosciences.com)

The assay was carried out according to the manufactures protocol without any modifications.

5.4. Results

The results obtained were generated in graphical and tabular format using the BD CBA analysis software and is summarised in the following figures:

- Figure 5.4 (a). IL-8 production by stimulated macrophages
- Figure 5.4 (b). IL-8 production by stimulated HUVEC
- Figure 5.5 (a). IL-1β production by stimulated macrophages
- Figure 5.5 (b). IL-1β production by stimulated HUVEC
- Figure 5.6 (a). IL-6 production by stimulated macrophages
- Figure 5.6 (b). IL-6 production by stimulated HUVEC
- Figure 5.7 (a). IL-10 production by stimulated macrophages
- Figure 5.7 (b). IL-10 production by stimulated HUVEC
- Figure 5.8 (a). TNF-α production by stimulated macrophages

- Figure 5.8 (b). TNF-α production by stimulated HUVEC
- Figure 5.9 (a). IL-12p70 production by stimulated macrophages
- Figure 5.9 (b). IL-12p70 production by stimulated HUVEC

Each of these graphs illustrates the results obtained from two control groups and eleven combinations of concentrations (AC represents Rimonabant and AEA represents anandamide). The thirteen groups were defined by the concentrations of the combination of the two compounds used to treat the cells as seen in Fig 5.3.

The group of wells treated with AC 0 µM and AEA 0 µM (Figure 5.3) produced the same results as the stimulated control group and is therefore not shown in the graphs.

The Kruskal-Wallis non-parametric test was used to determine statistical significance of the data relative to the stimulated control not treated with any of the test compounds. P values of less than 0.05 indicate significance compared to the relevant untreated stimulated control systems. Each value obtained represents the mean of 8 independent experiments. Several cytokine levels were not in the detectable range for the kit used during this experiment and were indicated on the graphs as no result.

The amount of each cytokine present in the sample is directly correlated to the fluorescent intensity emitted. Table 5.1 is a summary of the effect of Rimonabant and anandamide at different combination concentrations of the production of cytokines by human macrophages, as well as HUVEC.

Table 5.1: The production of cytokines by human macrophages and HUVEC after treatment with various concentrations of a combination of AC and AEA. M represents human macrophages, H represents HUVEC. The small ↑ represents a statistical insignificant increase, whereas the large ↑ represents a significant increase in cytokine production compared to the stimulated control. The small ↓ represents a statistical insignificant decrease, whereas the large ↓ represents a significant decrease in cytokine production compared to the stimulated control.

	M	H	M	H	M	H	M	H	M	H	M	H
	IL-8		IL-1β		IL-6		IL-10		TNF-α		IL-12p70	
AC 0 µM: AEA 5 µM	↓	↓	↓	↓	↓	**↓**	↓	↑	↓	↓	↓	↓
AC 0 µM: AEA 10 µM	↓	↓	↓	↓	↑	**↓**	↓	↑	↓	↓	↑	↓
AC 0.5 µM: AEA 0 µM	↓	↓	**↓**	↓	↓	↓	↓	↓	↓	↓	↑	↓
AC 0.5 µM: AEA 5 µM	↓	↓	↓	↑	↓	↓	↓	↓	↓	↓	↑	↓
AC 0.5 µM: AEA 10 µM	↓	**↑**	↓	↑	↓	↑	↓	↑	↓	↓	↓	↓
AC 1 µM: AEA 0 µM	↓	↑	↓	↓	↓	↑	↓	↓	↓	↓	↓	↓
AC 1 µM: AEA 5 µM	↓	**↑**	↓	↑	↓	↑	↓	↓	↓	↓	↓	↓
AC 1 µM: AEA 10 µM	↓	**↑**	↓	↑	↑	↑	↓	↑	↓	↓	↓	↓
AC 3 µM: AEA 0 µM	↓	↓	↓	↓	↓	↓	↓	↑	↓	↓	↓	↓
AC 3 µM: AEA 5 µM	↓	↑	↓	↑	↓	↑	**↓**	↑	↓	↓	↑	↓
AC 3 µM: AEA 10 µM	↓	↓	↓	↓	↑	↓	↓	↑	↓	↓	↑	↑

5.4.1. IL-8 production

LPS stimulated macrophages treated with each of the experimental compounds alone and various combinations of the compounds produced a decrease in the production of IL-8. According to Figure 5.4 (a), a small decrease in IL-8 can be observed at all the drug concentrations tested. However, none of these results were statistically significant.

No significant decrease in production of IL-8 by HUVEC was observed, however the following combinations:

- Rimonabant 0.5 µM: Anandamide 10 µM,
- Rimonabant 1 µM: Anandamide 5 µM and

- Rimonabant 1 µM: Anandamide 10 µM,

significantly increased the production of the pro-inflammatory cytokine, IL-8, compared to stimulated untreated control groups (Figure 5.4 (b)).

5.4.2. IL-1β production

The results indicated that the stimulated macrophages treated with Rimonabant at 0.5 µM significantly inhibited the production of the pro-inflammatory cytokine IL-1β (Figure 5.5 (a)). Stimulated HUVEC treated with various concentrations exerted no significant effect on the production of IL-1β when compared to the stimulated untreated control group (Figure 5.7). Rimonabant and anandamide on their own had no significant effect on the IL-1β levels of HUVEC. The combination of Rimonabant 1 µM with anandamide 5 µM, as well as the combination of Rimonabant 1 µM with anandamide 10 µM increased IL-1β levels slightly, but these results were not statistically significant.

5.4.3. IL-6 production

No significant differences in the levels of IL-6 were observed in LPS stimulated macrophage cultures after treatment with either Rimonabant or anandamide alone or in combination at the concentrations used in these experiments (Figure 5.6 (a)).

A significant decrease in the levels of IL-6 was observed after LPS stimulated HUVEC were treated with concentrations of 5 µM and 10 µM of anandamide (Figure 5.9) whereas Rimonabant had no effect on the production of this cytokine either on its own or in combination with AEA.

5.4.4. IL-10 production

The results obtained during this experiment indicated a decrease in the production of IL-10 by macrophages treated with both compounds alone or in combination (Figure 5.7 (a)). There was a significant reduction of IL-10 after treating macrophages with a combined concentration of Rimonabant at 3 µM and anandamide at 5 µM.

Neither Rimonabant nor anandamide alone or in combination at the concentrations used in these experiments had any effects on IL-10 production by LPS-stimulated HUVEC (Figure 5.11).

5.4.5. TNF-α production

The results indicate that Rimonabant and anandamide alone or in combination at the concentrations used caused a small reduction in the production of TNF-α by both macrophages and HUVEC (Figure 5.8 (a) & (b)). However, none of these results proved to be statistically significant.

5.4.6. IL-12p70 production

Neither Rimonabant nor anandamide alone or in combination at the concentrations tested had any effects on IL-12p70 production by LPS-stimulated macrophages (Figure 5.14) or HUVEC (Figure 5.15).

Figures 5.4 to 5.15 indicate the level (pg/ml) of cytokines present in the supernatants of cell cultures after a 48-hour treatment period.

5.5. Discussion

A BD Cytometric Bead Array Ready-to-use human inflammation kit was used during this experiment to simultaneously determine the levels of cytokine production by LPS stimulated human macrophages and HUVEC. Cytokines are a diverse group of bioactive proteins produced by various cell types including endothelial cells, macrophages, lymphocytes and neutrophils, and LPS is commonly used to stimulate the production of inflammatory cytokines *in vivo* and *in vitro* (Ao *et al*., 2006). One important feature of the inflammatory response is the elevation of pro-inflammatory cytokines i.e. TNF-α, IL-1β and IL-6 in blood and oedema fluid following tissue injury.

5.5.1. IL-8 production

The results obtained during this experiment indicated an increase in the production of IL-8 by macrophages and HUVEC in the controls stimulated with LPS for 24 hours. This

correlates with the findings of Miyamoto *et al*. (2002). Miyamoto *et al*. (2002) found that the IL-8 cytokine levels in the supernatant of cultured HUVEC reached peak levels after 12 hours exposure to the stimulant, Bleomycin.

As shown in Figure 5.4 (a) and (b), anandamide dose-dependently reduced LPS-induced production of IL-8 in both macrophages and HUVEC at concentrations of 5 µM and 10 µM. This result confirms the results obtained by Nakajima *et al*. (2006). Nakajima *et al*. (2006) investigated whether anandamide affected LPS-induced cytokine production in human gingival fibroblasts. It was concluded that anandamide concentrations of 1 µM, 5 µM and 10 µM exhibited a dose-dependent reduction in the production of IL-8 in these fibroblasts.

A different combinational effect was observed in the supernatant of cultured primary macrophages. The combination of 0.5 µM Rimonabant and 5 µM anandamide and 3 µM Rimonabant and 5 µM anandamide slightly reduced the production of IL-8, although these findings were not statistically significant.

As illustrated in Figure 5.4 (a), there was a slight reduction in levels of IL-8 detected following treatment with a combination of AC and AEA. The levels of IL-8 dropped to a non-detectible concentration in one test compound combination and was possibly due to loss of the beads during the workup, rather than a true decrease in production of this cytokine as no other combination showed an effect that matched such a large decrease in IL-8 expression.

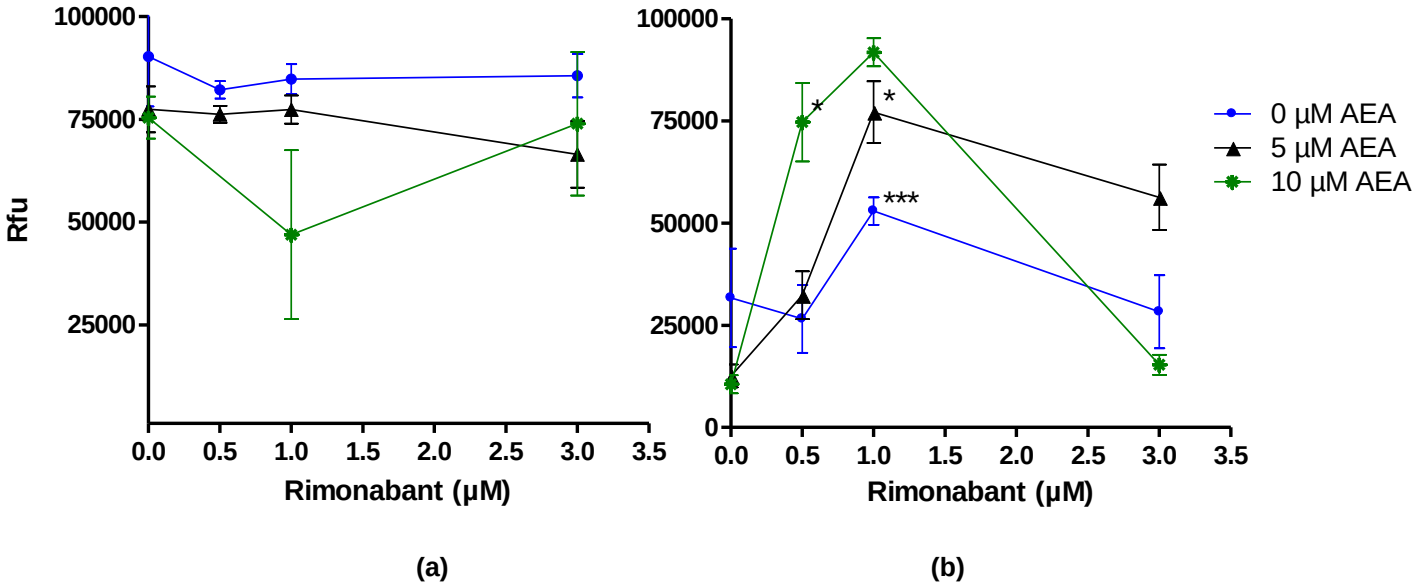


Figure 5.4. The IL-8 level (Rfu) present in the supernatant of (a) treated macrophages and (b) treated HUVEC.

The results using HUVEC indicated that adding the selective antagonist of the cannabinoid CB_1 receptor (Rimonabant), attenuated the effect of AEA. As shown in Figure 5.4 (b), there is a significant, dose-related increase in the production of IL-8 by HUVEC following treatment with a combination of Rimonabant and anandamide.

The data collected in Figure 5.4 (b) appears to be a bell-shaped dose-response curve with respect to the concentration of Rimonabant present in the culture media. These observations indicate that the optimal pro-inflammatory concentrations of Rimonabant are at the top of the bell-shape curve, which is in this case will be 1 µM and only when combined with anandamide. Rimonabant appears to demonstrate anti-inflammatory properties when used alone.

It is apparent from the results that Rimonabant at a concentration of 0.5 µM and anandamide (at all concentrations tested) have an anti-inflammatory effect on both macrophages and HUVEC when used alone. However, one exception was that Rimonabant had a pro-inflammatory effect on HUVEC at a concentration of 1 µM when combined with anandamide.

5.5.2. IL-1β production

The concentration of IL-1β present in the supernatant of the LPS stimulated macrophages and stimulated HUVEC was elevated when compared to the resting cell supernatant. This is in agreement with the results found during a study conducted on human mononuclear cells stimulated with LPS (Zurier *et al*., 2003).

As presented in Figure 5.5 (a) and (b), both macrophages and HUVEC showed a decrease in the production of IL-1β following treatment with 5 µM and 10 µM of anandamide respectively. This inhibitory effect was reversed during combination treatment with 1 µM of Rimonabant, as was seen with IL-8.

Zurier *et al*. (2003) showed that the cannabinoid THC-11-oic acid inhibited of IL-1β expression by human monocytes at all the concentrations tested (1 µM, 3 µM, 6 µM, 10 µM and 30 µM). This study showed similar results for both macrophages and HUVEC with Rimonabant treatment at concentrations of 0.5 µM and 3 µM (Figure 5.5 (a) and (b).

The results illustrated that anandamide inhibits the production of the IL-1β by both macrophages and HUVEC when present in the culture media. Rimonabant produced significant anti-inflammatory effects when used alone at a concentration of 0.5 µM. The combination concentrations of the two cannabinoid ligands appear to have a bell-shape dose-response when exposed to HUVEC. Rimonabant (1 µM) combined with different concentrations of anandamide had a pro-inflammatory effect on cultured HUVEC.

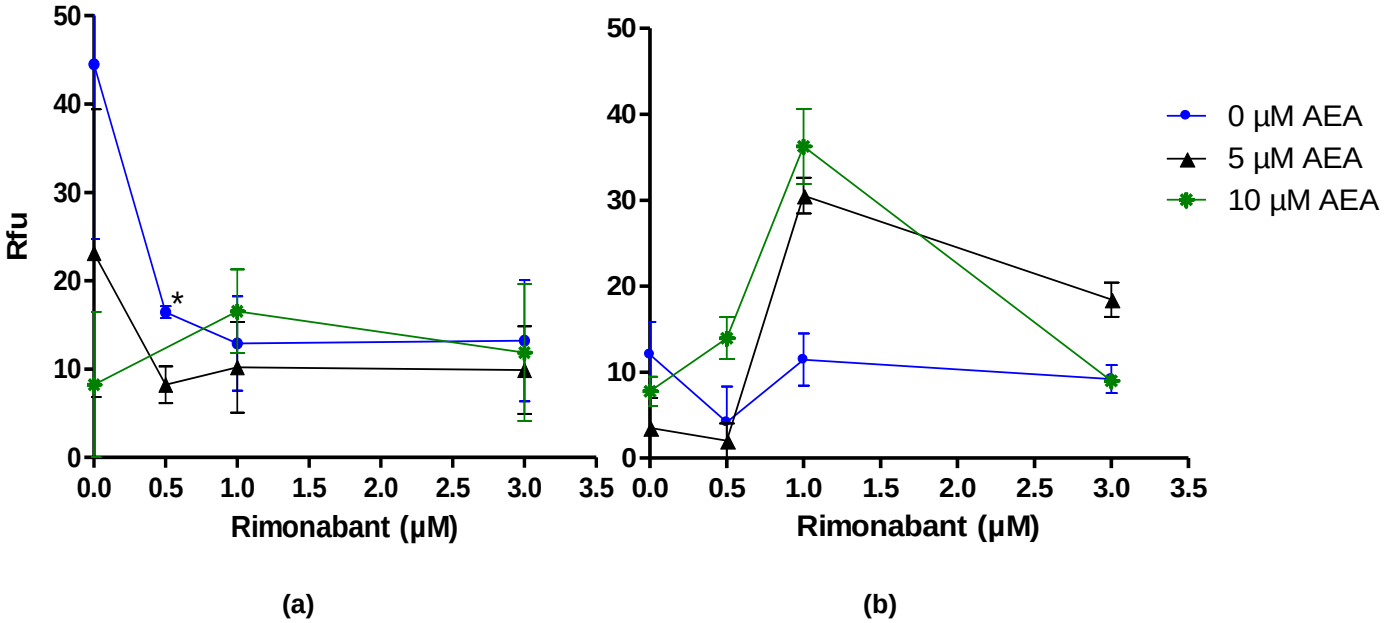


Figure 5.5. The IL-1β level (Rfu) present in the supernatant of (a) treated macrophages and (b) treated HUVEC.

5.5.3. IL-6 production

The results illustrated in Figure 5.6 (a) and (b) indicated that there was an increase in the production of IL-6 by stimulated macrophages and stimulated HUVEC, although the LPS induced cytokine production observed from the human macrophages were only small increases averaging approximately 200 pg/ml. These results are similar to the 150 pg/ml LPS induced increase of IL-6 observed during a study conducted on human gingival fibroblasts (Nakajima *et al.*, 2006) and the non-detectable IL-6 levels observed during a study on LPS stimulated murine macrophages (Ao *et al*., 2006).

A statistically significant, dose-dependent inhibitory effect of IL-6 production by HUVEC was observed after treatment with 5 µM and 10 µM of anandamide (Figure 5.6 (b)), although anandamide at these tested concentrations had no significant effect on the production of IL-6 by macrophages (Figure 5.6 (a)). Nakajima *et al.* (2006) published results indicating that anandamide at a concentration of 5 µM and 10 µM had a dose-related inhibitory effect on the production of IL-6 by human gingival fibroblasts, which is similar to the results obtained during this experiment.

Nakajima *et al*. (2006) reported that by adding 1 µM of SR144528 (CB_1 antagonist) to human gingival fibroblasts, the IL-6 inhibitory effect observed with treatment of 10 µM of anandamide alone was reversed. During the study reported here, the treatment of 1 µM Rimonabant combined with 5 µM and 10 µM anandamide respectively attenuated the inhibitory effect of anandamide observed during treatment of HUVEC. This combination treatment however, had insignificant effects on the production of IL-6 by stimulated macrophages.

Rimonabant inhibited the production of IL-6 by HUVEC at 0.5 µM and 3 µM, once again following the bell-shaped response. The effects of Rimonabant on the production of IL-6 by macrophages were inconsequential.

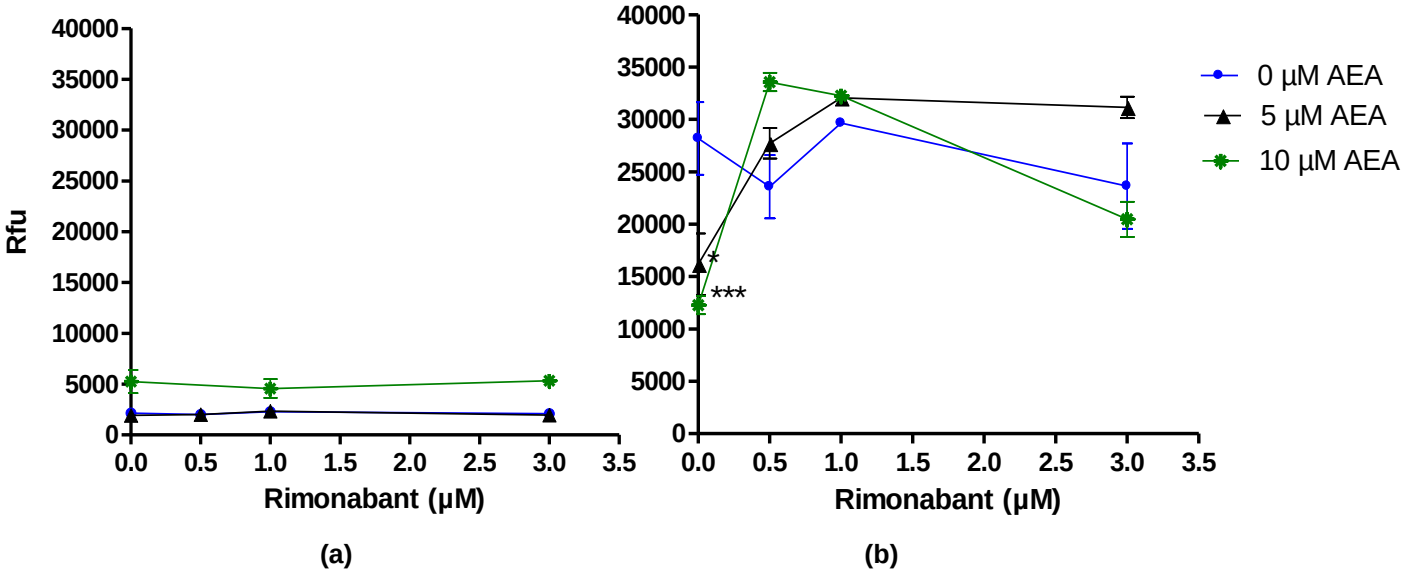


Figure 5.6. The IL-6 level (Rfu) present in the supernatant of (a) treated macrophages and (b) treated HUVEC.

5.5.4. IL-10 production

It is well known that macrophages produce very low concentrations of IL-10 (Ao *et al.*, 2006) and therefore the low levels observed in Figure 5.7 (a) were expected. HUVEC proved to produce even less IL-10 compared to the human macrophages and produced barely detectable levels of this cytokine (Figure 5.7 (b)).

Anandamide induced a dose related inhibitory effect on the production of IL-10 by macrophages at the concentrations tested (5 µM and 10 µM) and as with the other tested cytokines, Rimonabant (1 µM) attenuated the inhibitory effect anandamide had on macrophages (Figure 5.7 (a)).

Rimonabant inhibited IL-10 expression into the supernatant of cultured macrophages at concentrations of 0.5 µM and 3 µM, which is similar to the results obtained for the other tested cytokines (Figure 5.7 (a)). The low levels of IL-10 present in the medium of the cultured HUVEC limits the conclusions that can be drawn from these results (Figure 5.7 (b)).

Due to the extremely low levels of IL-10 detected the results for this cytokine should not be regarded as reliable and it was thought that if a definite trend could be detected that the experiment would have been repeated using larger volumes of the supernatant. However, the changes observed were insignificant and relatively random, not showing any trends.

There were again some samples where the capture beads appear to have been lost during the assay for one combination of drug in both the macrophage and HUVEC assays.

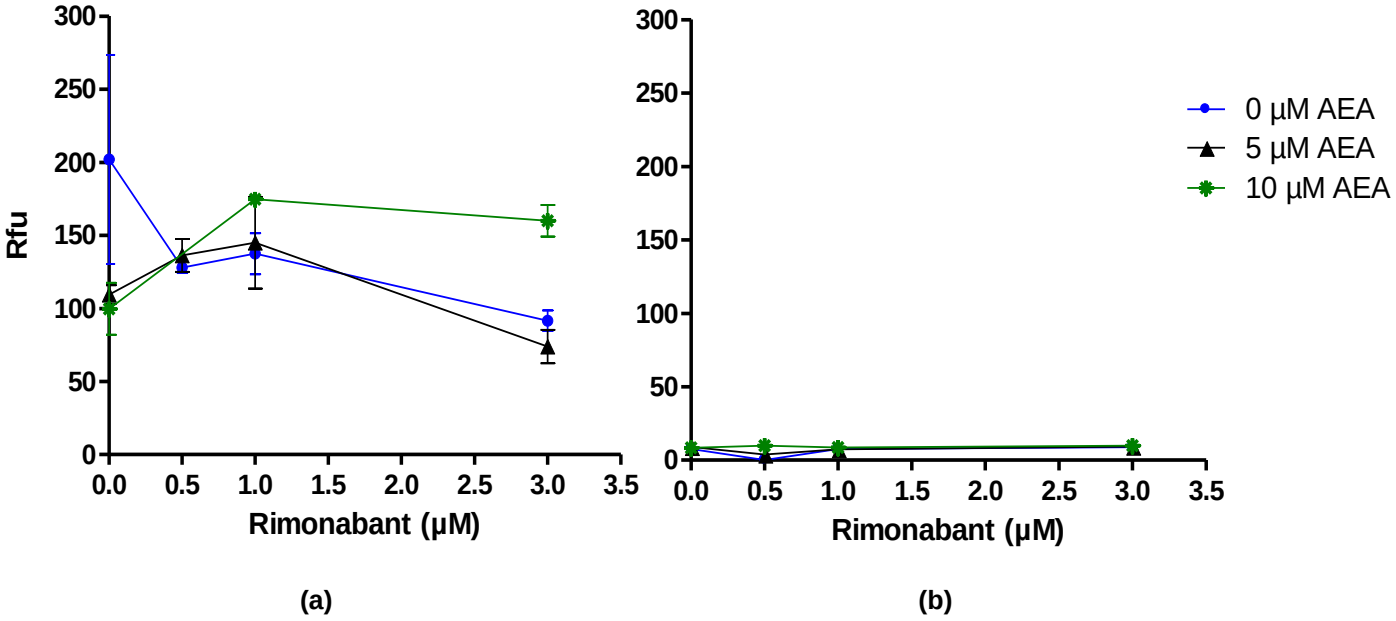


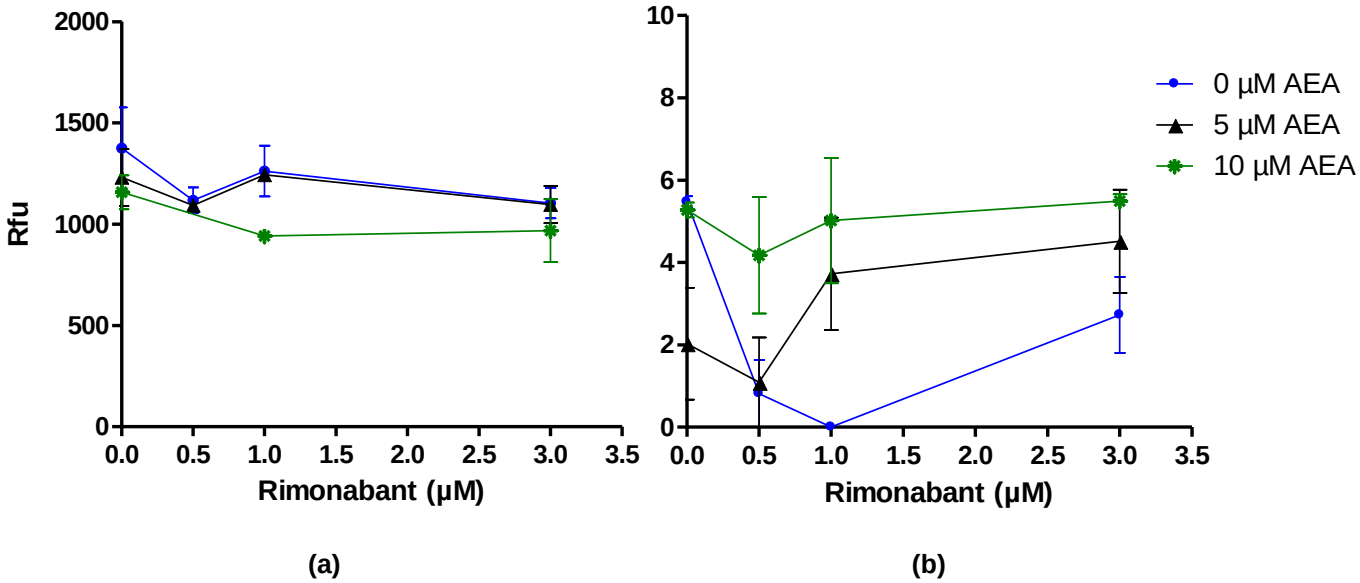
Figure 5.7. The IL-10 level (Rfu) present in the supernatant of (a) treated macrophages and (b) treated HUVEC.

5.5.5. TNF-α production

According to Ao *et al*. (2006), no TNF-α is detectable in the culture media of resting murine macrophages, but stimulation with LPS causes an increase in the concentration of the cytokine from non-detectable to approximately 1283 pg/ml after the 11th hour of stimulation. Thus a similar result was expected during this study evaluating the production of TNF-α by human macrophages. Even though LPS stimulation increased the production of TNF-α by HUVEC, the concentrations of this cytokine was very low and of no significance (Figure 5.8 (b)).

No significant effect on the production of TNF-α was observed after treatment with all the various concentration of anandamide and Rimonabant alone or in combination (Figure 5.8 (a)). These results correlate with the findings of Zurier *et al*. (2002), who reported that ajulemic acid (an analog of THC-11-oic acid) had no significant effect on the production of TNF-α by LPS stimulated peripheral blood monocytes. The results presented in Figure 5.12 (a) and (b), indicate that Rimonabant and anandamide had no effect on the production of TNF-α by ether human macrophages or HUVEC and taking

previous findings into account might suggest that cannabinoid ligands do not affect TNF-α production.

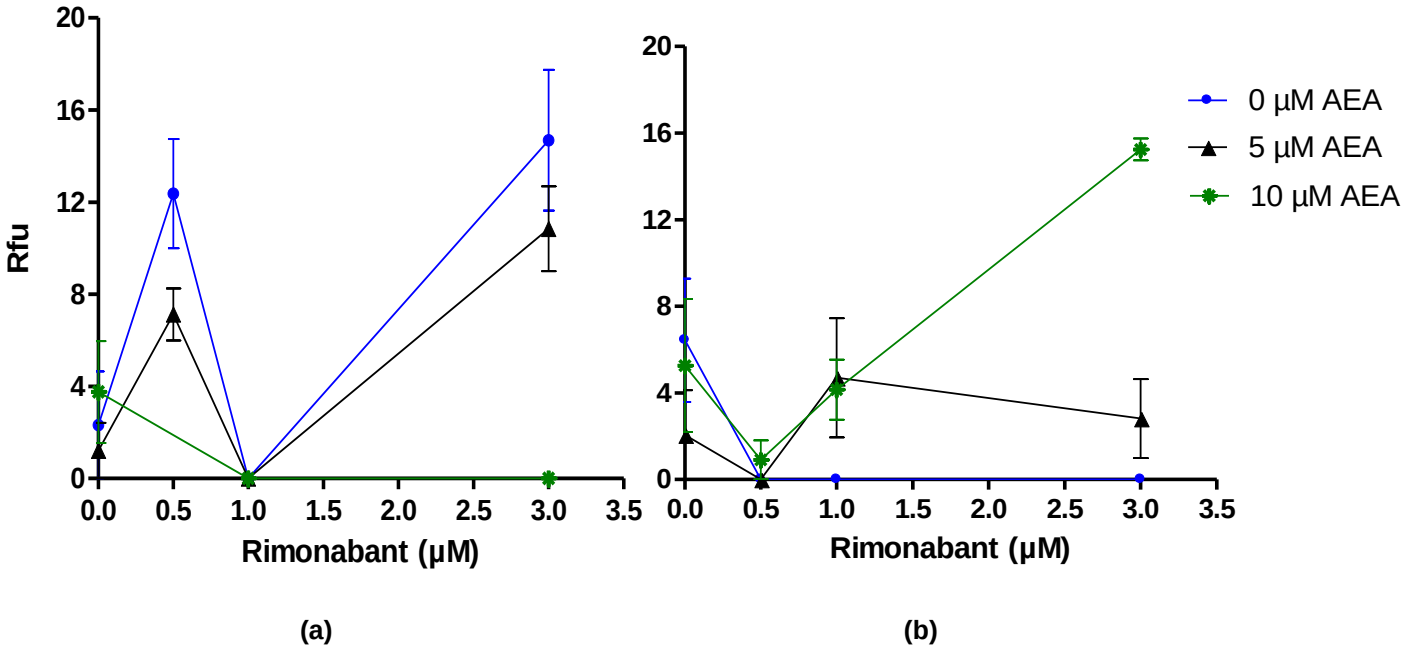


(a) **(b)**

Figure 5.8. The TNF-α level (Rfu) present in the supernatant of (a) treated macrophages and (b) treated HUVEC. Note the small scale on the Y axis due to the extremely low levels of TNF-α expressed by the HUVEC.

5.5.6. IL-12p70 production

The results presented in Figure 5.9 (a) and (b), indicate that there were extremely low levels of IL-12p70 produced by human macrophages as well as HUVEC. This result imitated the non-detectable ranges reported by Ao *et al*. (2006), following the stimulation of murine macrophages. None of the results obtained regarding IL-12p70 production were statistically significant.



(a) (b)

Figure 5.9. The IL-12p70 level (Rfu) present in the supernatant of (a) treated macrophages and (b) treated HUVEC.

5.6. Conclusion

The results showed that both human macrophages and HUVEC produced IL-8, IL-1β and IL-6 *in vitro*, however IL-12p70 was produced at such low levels in the culture media aliquots, that it was impossible to effectively evaluate the effects of the compounds on this cytokine. HUVEC produced very low concentrations of IL-10 and TNF-α, which made meaningful evaluation difficult.

The endocannabinoid anandamide was shown to inhibit the production of all the detectable cytokines (IL-8, IL-1β and IL-6 in both cell types and IL-10 and TNF-α in macrophages). Furthermore this inhibitory effect was attenuated by pre-treatment with a potent, selective antagonist of the cannabinoid CB_1 receptor, namely Rimonabant. These results would suggest that anandamide could induce anti-inflammatory effects observed in macrophages and HUVEC, through cannabinoid receptors.

Rimonabant also inhibited the production of all the detectable cytokines (IL-8, IL-1β and IL-6 in both cell types and IL-10 and TNF-α in macrophages) following treatment with

0.5 µM and 3 µM respectively, but only IL-1β was significantly reduced in the supernatant of cultured macrophages pre-treated with 0.5 µM Rimonabant. The anti-inflammatory effects of anandamide were attenuated when combined with 1 µM of Rimonabant. Throughout the various cytokine responses, the dose-response relationship appeared to follow a bell-shaped dose-response. This response was characterised by high inhibitory effect of cytokine expression compared to control at low inhibitor concentrations (0.5 µM), with a significant increase in cytokines at mid-range inhibitor concentrations (1 µM), followed by a decrease of cytokines to below control levels at higher inhibitor concentrations (3 µM). This occurrence proposes that Rimonabant displaces anandamide and blocks the anti-inflammatory effects of the agonist. Once the concentration of antagonist that can completely displace the agonist is reached, the inverse-agonistic effects of Rimonabant are observed. Rimonabant binds to the same receptor as anandamide, but induces a pharmacological response opposite to that of anandamide and increases the production of cytokines above the basal level. The peak pro-inflammatory response of Rimonabant is reached at a concentration of approximately 1 µM. Further increase in concentration of Rimonabant was associated with a decrease in the magnitude of inverse agonistic effects and subsequent return to baseline response, resulting in the bell-shape dose-response curve.

This sharp decrease in the production of cytokines at high concentrations of Rimonabant might be ascribed to an effect on a different receptor. Even though Rimonabant is a highly selective CB_1 antagonist/inverse agonist, it does not exclude the possibility of slight affinity for the CB_2 or an alternative receptor. It is possible that during saturation of the CB_1 receptors, Rimonabant binds to CB_2 receptors and activates the anti-inflammatory response associated with this cannabinoid receptor. These results highlight the immunological significance of the two functional cannabinoid receptors and immuno-pharmacodynamics can potentially be changed across the different concentrations of immunologically active ligands.

Knowing the vital role that these pro-inflammatory cytokines play in immune and inflammatory responses, this finding suggest that one of the mechanisms by which

Rimonabant and anandamide inhibits inflammatory reactions is by acting on the cytokine producing cells. The exact method and receptor involved is still unknown and requires further experimentation, at varying sub-saturated concentrations of CB_1 receptors and potentially different incubation times, to fully understand this anti-inflammatory response. A variety of cell types and culturing methods would offer additional clarity, since the effect of Rimonabant and anandamide has been shown to be dependent upon the cell type employed.

Chapter 6: ICAM-1 expression by HUVEC

6.1. Introduction

This chapter is focused on determining the effect of Rimonabant (AC) and anandamide (AEA) (), alone or used in combination, on the expression of the intercellular adhesion molecule-1 (ICAM-1) by HUVEC. ICAM-1 expression is a key step in the inflammatory response of the body. If Rimonabant, used in combination with anandamide (which is naturally present in the human body), inhibits the expression of ICAM-1, Rimonabant could be used as an adjuvant when developing new anti-inflammatory drugs.

The immune system consists of several powerful defence mechanisms to guard the host against possible pathogen invasion that would otherwise benefit from the rich source of nutrients provided by the host. The human immune response is primarily mediated by leukocytes (Male *et al*., 2006). One important step in leukocyte recruitment during a localised inflammatory response is the adhesion of specific leukocytes to the vascular endothelium followed by movement into the extra-vascular tissue. Adhesion of leukocytes to endothelial cells occurs through the binding of intercellular adhesion molecules (ICAMs) on the endothelium to heterodimeric proteins of the integrin family on the surface of activated leukocytes (Figure 6.1) (Janeway *et al*., 2005). This adhesion of leukocytes is the first in a controlled sequence of events that leads to diapedesis where the leukocytes move into the extra-vascular tissue. The CAMs are all members of the immunoglobulin supergene family and are expressed on the surface of endothelial cells and act as ligands for surface expressed leukocyte integrins. The CAMs include, ICAM-1, ICAM-2, vascular cell adhesion molecules-1 (VCAM-1) and mucosal addressin CAM (MAdCAM-1) (Male *et al*., 2006). Several adhesion molecules are produced and expressed on the luminal surface of vascular endothelial cells in response to inflammatory stimuli and these include: ICAM-1, VCAM-1, E-selectin and P-selectin (Figure 6.1) (Nizamutdinova *et al*., 2006).

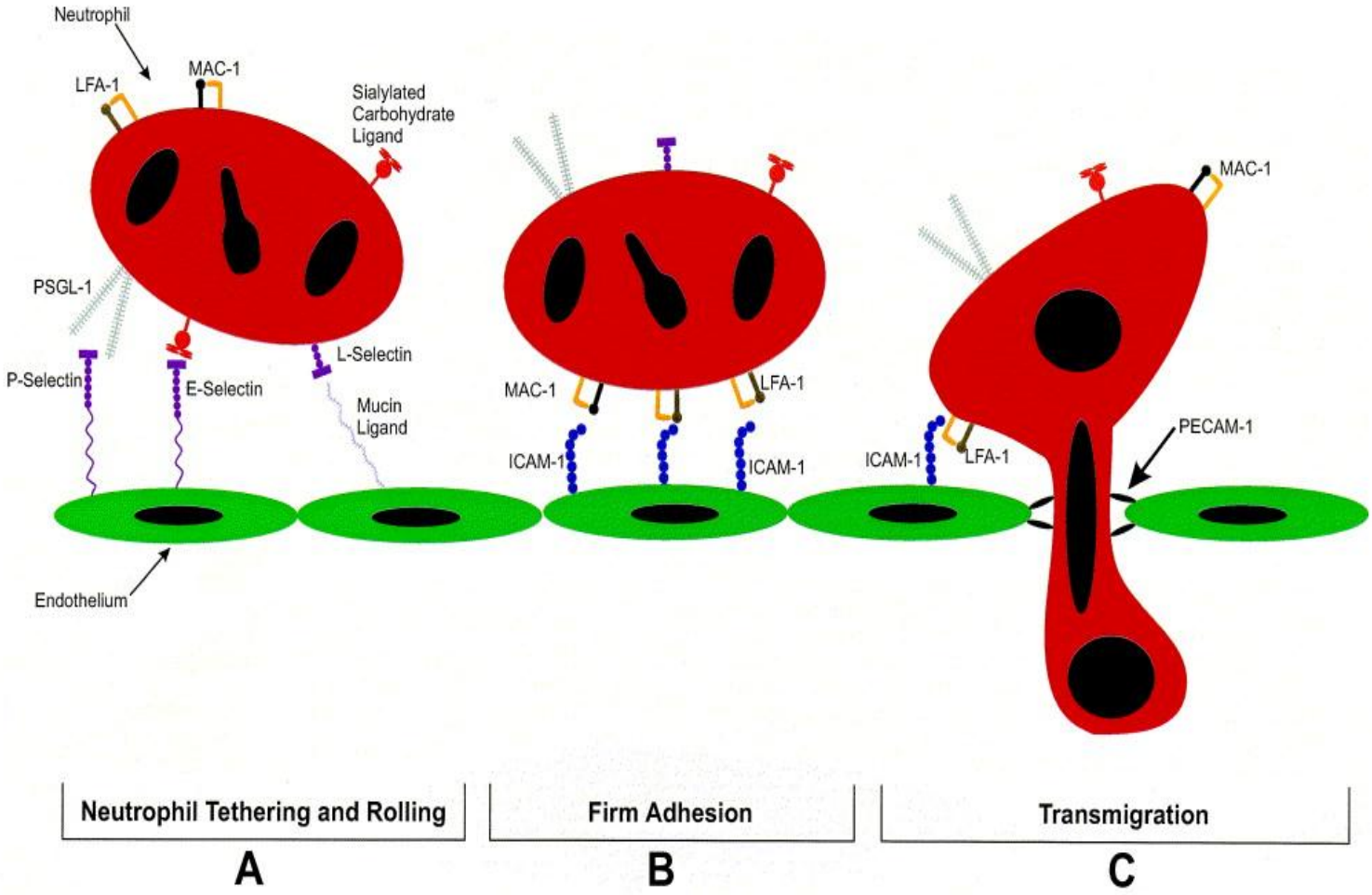


Figure 6.1. The role of the endothelial cell and ICAM in the resolution of acute inflammation (Meager, 1999)

Adhesion molecules play a vital role in cell-to-cell interactions, as well as cell-to-extracellular matrix interactions (Hou *et al*., 2005). The two important leukocyte integrins involved in the adhesion response are leukocyte functional antigen 1 (LFA-1) and compliment receptor 3 (CR3) both of which bind to ICAM-1 (CD54) and ICAM-2. During an inflammatory response, macrophages released cytokines, such as TNF-α, IL-1β, IFNγ and damaged bacteria release lipopolysaccharides (LPS), all of which induce the expression of ICAM-1 by endothelial cells of small vessels near or within an infected area. ICAM-1 then binds LFA-1 and CR3 on the surface of activated circulating monocytes and PMN and a strong adhesion between leukocytes and endothelial cells occurs (Figure 6.1) (Janeway *et al*., 2005). This adhesion is the first step in diapedesis,

the process whereby inflammatory cells traverse the vascular wall leading to inflammation within the adjacent tissue (Figure 6.1) (Nizamutdinova *et al*., 2006).

Bucillamine [N-(2-mercapto-2-methylpropionyl)-L-cysteine] (BUC) is used in the treatment of rheumatoid arthritis and this disease-modifying anti-rheumatic drug has several beneficial effects during treatment (Kikuchi *et al.*, 2003). In a study done by Kikuchi *et al.*, 2003, it was found that an intramolecular disulfide form of BUC called BUC-ID inhibits the expression of VCAM-1 on HUVEC at pharmacologically relevant concentrations.

In a study done on murine brain endothelial cells infected with Theiler's murine encephalomyelitis virus (TMEV), a CB_1/CB_2 receptor agonist (WIN55,212-2) was found to down-regulate the expression of ICAM-1 and VCAM-1 adhesion molecules in the brain. It was also found that early treatment with WIN55,212-2 interfered with the development of TMEV-induced demyelinating disease in mice. Increasing evidence indicate that adhesion molecules present in neurovascular endothelium are involved in multiple sclerosis development and by inhibiting the expression of these adhesion molecules, multiple sclerosis symptoms could possibly be reduced or even prevented (Mestre *et al*., 2009).

According to Collins *et al*. (2004), depleting the gene for ICAM-1 in apolipoprotien E-deficient mice resulted in a significant reduction in monocyte recruitment at atherosclerotic lesions and protected against atherosclerosis. This emphasises the importance of ICAM-1 in the development of atherosclerosis which might be involved in the development of many other autoimmune diseases.

Increased expression of adhesion molecules on vascular endothelial cells is one of the key steps in the accumulation of inflammatory cells. Initially, monocytes adhere to activated endothelium presenting cell adhesion molecules on their surface, before migrating across the endothelial layer into the tissue where they differentiate into macrophages (Stannard *et al*., 1998).

The inhibition of cell adhesion molecules has been shown to be a useful therapeutic approach to control acute and chronic inflammatory diseases. Additionally, previous findings suggest that the cannabinoids have the ability to inhibit adhesion molecule expression (Mestre *et al*., 2009). ICAM-1 inhibition could thus be a useful cellular target for pharmacologically based immune modulation.

6.2. Aim

The aim of this study was to determine the *in vitro* effects of Rimonabant (AC) and anandamide (AEA) alone and in combination on the extracellular surface expression of ICAM-1 by HUVEC.

6.3. Materials and methods

This method makes use of the CD54-FITC monoclonal antibody and flow cytometry to quantitate the expression of ICAM-1. This method was repeated 6 times with the HUVEC from 6 different umbilical cord donors to obtain the results in Figure 6.2. Eleven different drug combinations, using Rimonabant at 0.5 µM, 1 µM, 3 µM and anandamide at 5 µM, 10 µM, as well as two control groups was used in this investigation as indicated below in Figure 6.2. These combination concentrations are similar to the concentrations of anandamide combined with selective CB_1 and CB_2 antagonists used by Nakajima *et al*. (2005).

6.3.1. Preparation of reagents

All the prepared reagents were sterilised before use and stored in sealed sterile containers. The containers were only opened inside a sterilised laminar flow cabinet (Labotec, model LCA 6000VA) to prevent contamination of the reagents. 199 medium+ was prepared according to the method described in Section 2.3.1 and the cell counting fluid was prepared according to the method described in Section 3.3.2. Rimonabant and anandamide were prepared according to the method described in Section 3.3.1.

i. Interleukin 1 Beta (IL-1β)

Recombinant Human Interleukin 1 beta, (*E. coli*-derived), was purchased from R&D Systems in 5 µg units. IL-1β (5 µg) was dissolved in 1 ml of 199 medium+ and was stored in 10 µl aliquots at –70^{o}C until needed. A 10 µl aliquot was diluted in 4990 µl of 199 medium+ and further diluted as a 1:9 dilution upon addition to cells (50 µl of IL-1β dilution into 450 µl of cell suspension). The final concentration was 1 ng/ml which is the same concentration used by Imaizumi *et al*. (2000) on HUVEC cultures. In a study done by Kiszel *et al.* (2007), IL-1β was tested in different concentrations on HUVEC to determine the optimal concentration for stimulation. Kiszel *et al.* (2007) concluded that 1 ng/ml of IL-1β proved to be effective to stimulate HUVEC.

ii. Monoclonal antibody

An antibody against human CD54-FITC (fluorescein isothiocyanate conjugated), purchased from Beckman Coulter, was used during this experiment. Non-specific binding was excluded by inclusion of a relevant mouse IgG-PE isotypic control.

6.3.2. Experimental procedures

Twenty four sterile glass tubes were labelled as follows: Resting isotypic control, stimulated isotypic control, resting control, stimulated control, resting (various drug concentrations) and stimulated (various drug concentrations). The glass tubes were used to ensure that HUVEC did not attach to the bottom of the tubes and thus ensured that they stayed in suspension to be read by the flow cytometer. The HUVEC were gently scraped from the bottom surface of the culture flask using a sterile cell scraper. The HUVEC were then placed into a sterile 5 ml centrifuge tube and centrifuged for 8 minutes at 200 *g*. The supernatant (containing the 199 medium+) was removed without disturbing the pellet (containing the HUVEC). The pellet was resuspended into 1 ml 199 medium+ and counted as described in Section 3.3.3 iii. The cells were then diluted to a 1 x 10^{5} cells/ml concentration in the same medium. This uniform cell suspension was then placed into each of the differently labelled glass tubes (250 µl in each tube). The glass tubes were sealed and incubated in a 37^{o}C water bath for 2 hours. After the incubation period Rimonabant (100 µl of the various concentrations) and anandamide (100 µl of the various

concentrations) was added to the appropriately labelled tubes. The tubes were sealed and incubated in a 37^{o}C water bath for 15 minutes (the control tubes received 200 µl 199 medium+). IL-1β (50 µl) was added to the stimulated tubes and 50 µl of 199 medium+ was added to the resting tubes. The tubes were then incubated for 24 hours in a 37°C/5% CO_2 incubator.

The monoclonal antibody (CD 54-FITC) was dispensed into corresponding (labelled the same as the glass tubes) sample acquisition tubes for use in a flow cytometer (20 µl of the antibody in each tube). An aliquot of cell suspension from the glass tubes (100 µl) was added to the respective acquisition tubes containing the antibody and incubated for 10 minutes in the dark. Isoflow (400 µl) was added to dilute the cell/antibody mix and analysed for FITC fluorescence using a Beckman Coulter FC500 flow cytometer.

6.4. Results

The results are expressed as the mean percentage of untreated controls ± SEM. Each value represents the mean of 4 independently repeated experiments. The mean and graphs were obtained using the GraphPad Prism 4 software package. The P-values indicate a comparison between the experimental values and the control values and were obtained using the Kruskal-Wallis test. A minimum of 1000 events in the gated population of cells was counted for each sample and the fluorescence measured by the flow cytometer was quantified as mean fluorescence intensity units (Figure 6.2).

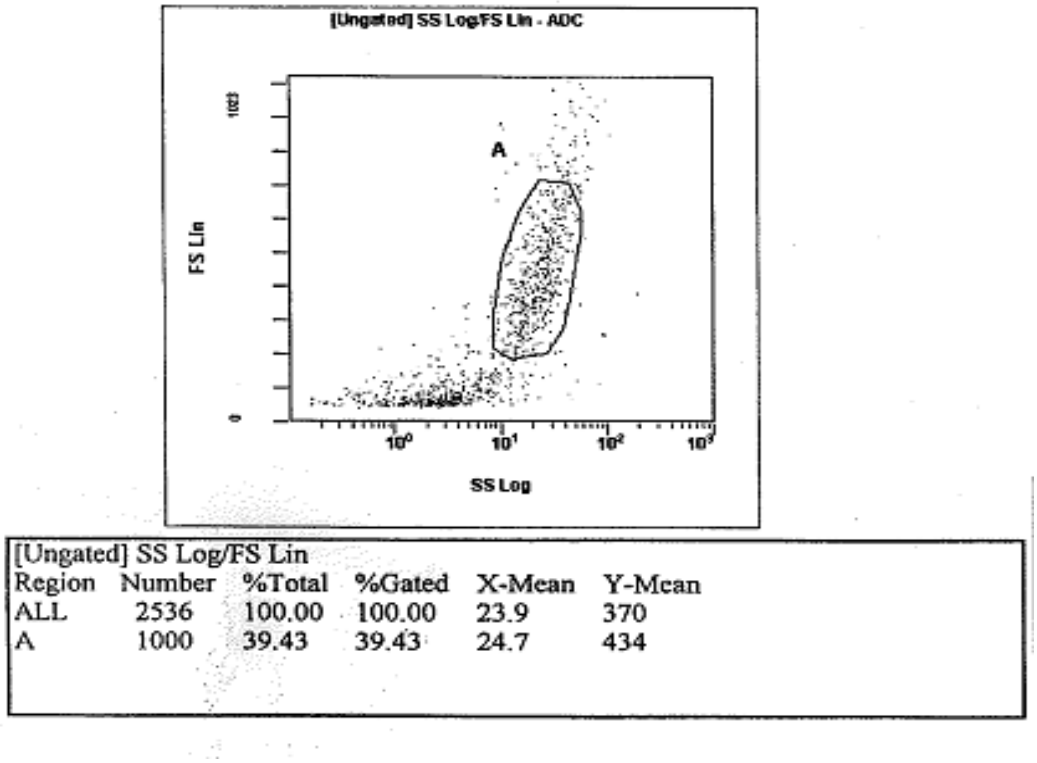


Figure 6.2. Flow cytometric analysis of HUVEC for the expression of ICAM-1 following staining with CD 54-FITC. The gated region represents the CD54 positive cell population.

The results were presented in graphical format as shown in Figure 6.3 and Figure 6.4. Figure 6.3 illustrates the results obtained from a control group and eleven different combination concentrations (AC represents Rimonabant and AEA represents anandamide). Twelve groups were defined by the different concentrations of the combination of the two compounds used to treat the cells. Each group was evaluated at resting (Figure 6.3) and IL-1β stimulated (Figure 6.4) conditions. The groups were as follows:

- Group 1 Control (no AC or AEA)
- Group 2 AC 0 µM: AEA 5 µM
- Group 3 AC 0 µM: AEA 10 µM
- Group 4 AC 0.5 µM: AEA 0 µM
- Group 5 AC 0.5 µM: AEA 5 µM

- Group 6 AC 0.5 µM: AEA 10 µM
- Group 7 AC 1 µM: AEA 0 µM
- Group 8 AC 1 µM: AEA 5 µM
- Group 9 AC 1 µM: AEA 10 µM
- Group 10 AC 3 µM: AEA 0 µM
- Group 11 AC 3 µM: AEA 5 µM
- Group 12 AC 3 µM: AEA 10 µM

The results summarised in Figure 6.3 and Figure 6.4 indicate that HUVEC responded to IL-1β stimulation with increased ICAM-1 expression on the cell surface, however treatment with Rimonabant and anandamide (alone and in combination) was unable to significantly inhibit or stimulate this effect.

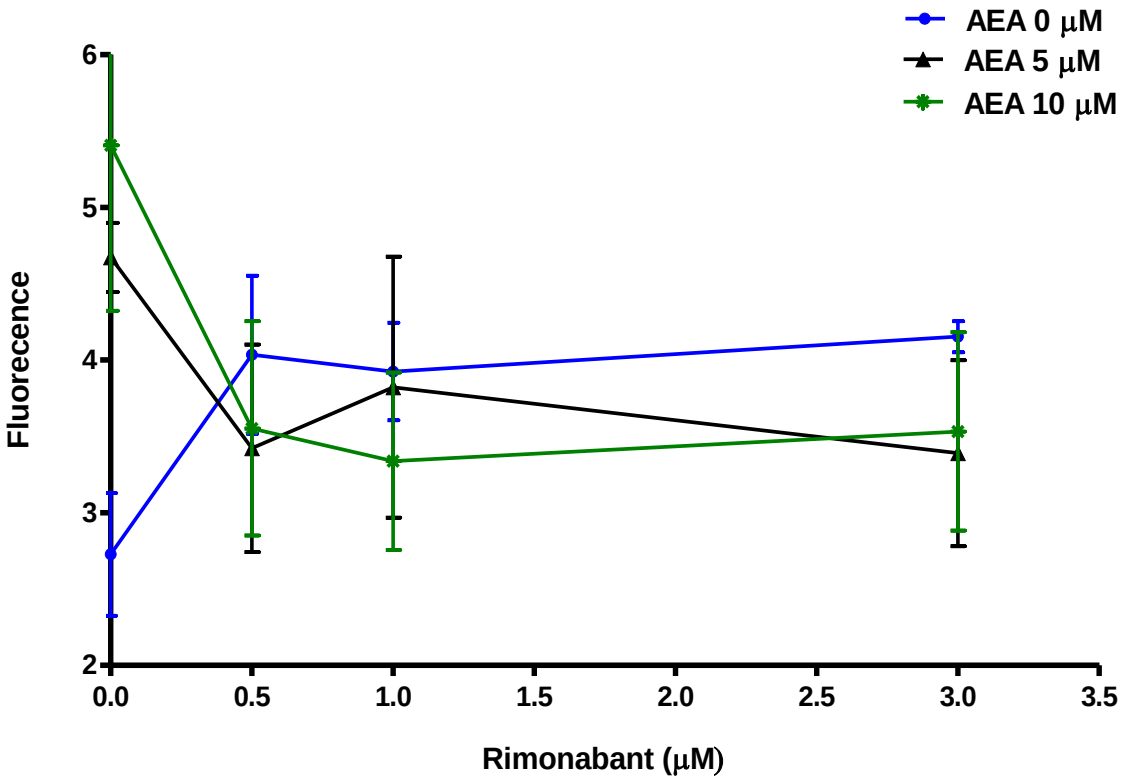


Figure 6.3. The effects of Rimonabant and anandamide, alone and in combination, on the ICAM1 expression of resting HUVEC (n = 4).

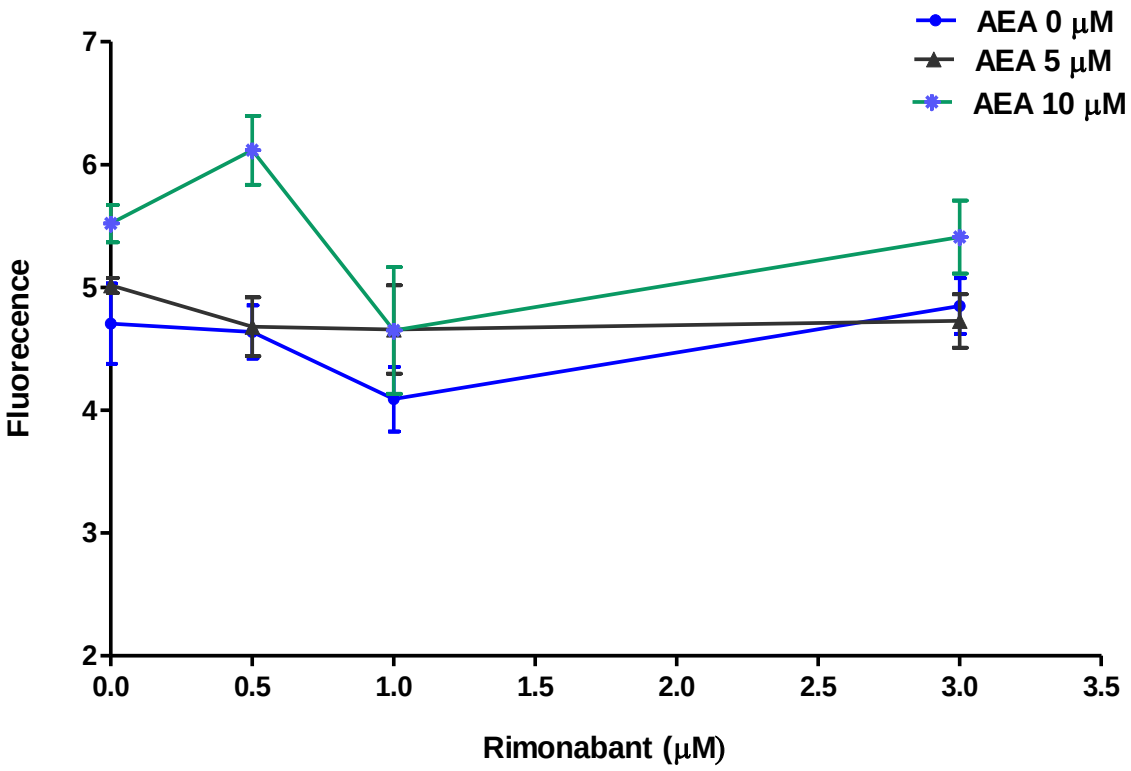


Figure 6.4. The effects of AC and AEA, alone and in combination, on the ICAM-1 expression of IL-1β stimulated HUVEC (n = 4).

6.5. Discussion

This *in vitro* experiment used a flow cytometer based assay to determine the relative changes of ICAM-1 (CD54) concentration on the surface of resting and IL-1β stimulated HUVEC resulting from treatment with Rimonabant and anandamide (alone and in combination). Adhesion molecule expression was assessed by assaying the intensity of a fluorescently FITC-conjugated monoclonal antibody (CD54), which was bound to cultured HUVEC, through the flow cytometer. An equivalent isotypic mouse immunoglobulin G (IgG) was used as the control antibody to assess non-specific binding. The flow cytometer can quantitate the florescence intensity emitted by individual labelled cells (Ormerod, 2008) and a direct staining method was selected due to its benefit of requiring only one antibody incubation step and it eliminates the possibility of non-specific binding from a secondary antibody. The intensity of the fluorescence emitted by

the cells in the samples was directly proportional to the amount of ICAM-1 molecules present on their membranes.

This experiment presents results indicating that there was an approximate two-fold increase in the expression of ICAM-1 by HUVEC following IL-1β stimulation for 24 hours (Figure 6.4). This result is comparable to the results obtained by Wheller *et al*. (1997). Using a similar method on two different human umbilical vein endothelial cell-derived cell lines called LT4 (SV40-transformed HUVEC) and ECV304 (spontaneously transformed HUVEC), Wheller *et al.* (1997) found that ICAM-1 expression was increased two-fold following a 24 hours stimulation period with IL-1β.

In resting and stimulated conditions, HUVEC expressed negligible levels of ICAM-1 on their cell surface compared to the results obtained by Wheller *et al.* (1997). The reason for these discrepancies is likely to be critical experimental differences between the studies. The first difference was the concentration of cells used during each experiment. Wheller *et al*. (1997) seeded the cells in 6-well plates at a concentration five-times greater than the concentration of HUVEC used during this experiment and at least 5000 events were recorded by the flow cytometer for each sample compared to the 1000 events counted during this experiment. Wheller *et al*. (1997) proceeded to stimulate these cell lines with 100 ng/ml of IL-1β compared to the 1 ng/ml used to stimulate the HUVEC during this experiment. The Wheller *et al*. (1997) publication compared the processing of ICAM-1 on the cell surface of different activated HUVEC-derived cell lines following IL-1β and TNF-α stimulation and found that the expression of ICAM-1 on their cell surfaces ranged from a two-fold increased for IL-1β to a five-fold increase for TNF-α. They established that TNF-α not only up-regulated ICAM-1 expression more affectively compared to IL-1β, but produced a time-dependent increase in ICAM-1 levels (up to one hundred-fold increase by 24 hours post-stimulation).

Kiszel *et al*. (2007) as well as Imaizumi *et al.* (2000) indicated that 1 ng/ml of IL-1β provided optimal stimulation of cultured HUVEC. Their results were however based on the optimal production of cytokines and not on the expression of cell surface molecules.

The activation of the ICAM-1 gene, which leads to the up-regulation of ICAM-1 protein expression, is a process requiring full functioning of the proteosome multicatalytic complex as well as the translocation of specific nuclear factors (including nuclear factor-kB) into the nucleus (Read *et al*., 1995). The fact that a higher presence of ICAM-1 was observed following 100 ng/mg of IL-1β suggests that a greater concentration of cytokine is required to activate the multiple factors operating to achieve ICAM-1 up-regulation on endothelial cells.

According to the results summarised in Figure 6.3 and Figure 6.4, neither Rimonabant nor anandamide had any significant inhibitory effect on the expression of ICAM-1 by HUVEC. None of the compound combinations tested during this experiment had any significant effects on the ICAM-1 expression on resting or IL-1β stimulated HUVEC. During a study done by Rajesh *et al*. (2007), it was concluded that the CB_1 antagonist (AM251) and the CB_2 antagonist (AM630) had no effect on the expression of ICAM-1 in TNF-α stimulated human liver sinusoidal endothelial cells (HLSECs). Rajesh *et al*. (2007) published results indicating that the cannabinoid antagonists had no effect on ICAM-1 expression, whereas the agonist, HU-308 (CB_2 receptor agonist) dose-dependently inhibited the expression of ICAM-1 in TNF-α treated HLSECs. These results correlate with the results presented in Figure 6.3 and Figure 6.4, indicating that the CB_1 receptor antagonist, Rimonabant had no significant effect on the expression of ICAM-1 by resting or stimulated HUVEC. However the lack of inhibitory action observed following treatment with the cannabinoid agonist, anandamide contradicts the findings presented in the Rajesh *et al.* (2007) publication.

This contradiction might be ascribed to the fact that anandamide is an endocannabinoid which might react differently compared to the synthetic compound, HU-308. It might be due to the different endothelial cells types used during the two different experiments or more likely the different stimulants used. TNF-α was the stimulant selected by Rajesh *et al*. (2007) and produced a five-fold increase in the production of ICAM-1 when compared to the resting control, similar to the Wheller *et al*. (1997) findings. Potentially the extent of the increase in the ICAM-1 expression observed during this study using IL-

1β stimulation could not provide a clear indication of the effect of anandamide and therefore also not the effect of anandamide in combination with Rimonabant on the expression of ICAM-1.

6.6. Conclusion

No significant inhibitory effect for Rimonabant or anandamide (alone and in combination) on the expression of ICAM-1 by either resting or IL-1β stimulated HUVEC. These findings suggest that the method of action by which Rimonabant exerts an anti-inflammatory action probably does not involve altering the surface expression of intracellular cell adhesion molecule-1 by primary cultures of HUVEC.

Considering the low levels for ICAM-1 expressed by the IL-1β stimulated HUVEC during this experiment and the literature supporting more effective methods of activating the ICAM-1 gene and subsequent up-regulation of ICAM-1 proteins, TNF-α stimulation of HUVEC might produce a different result. Larger quantities of ICAM-1 expression on the surface of HUVEC will provide more conclusive results and will aid in requiring further confirmatory results in order to finally rule out inhibition of ICAM-1 expression as a mechanism of action for AC (Rimonabant) and AEA (anandamide).

Chapter 7: Neutrophil adhesion to HUVEC

7.1. Introduction

This study investigated the possibility of Rimonabant inhibiting or preventing the adhesion of neutrophils to endothelial cells. The adhesion of neutrophils to the endothelium enables the neutrophils to move out of the vasculature into the inflamed area where they exert their effects. If the neutrophils were unable to adhere to the endothelium, they would be unable to exert their inflammatory action in the target area. If Rimonabant possess an inhibitory effect on the extravascularisation of neutrophils, it could provide information to elucidate the mechanism of the anti-inflammatory effect observed for this CB_1 antagonist.

Polymorphonuclear leukocytes or granulocytes are cells that are part of the first line of defence in the innate immune response. These granulocytes are divided into neutrophils, basophils and eosinophils (Austyn *et al*., 1993). Neutrophils are the most abundant white cells in the blood (around 60-70% in adults) and they are formed in the bone marrow (Nairn *et al*., 2002). Neutrophils provide an effective initial host defence against bacterial and fungal infections. Patients with defective or low levels of neutrophils (neutropenic) are at great risk of serious bacterial infections (Nairn *et al*., 2002). Neutrophils, as well as macrophages are phagocytic cells recruited to the site of inflammation in various tissues of the body. Human peripheral blood contains a central pool (circulating axial) of neutrophils and a marginal pool of neutrophils moving slowly along the vascular endothelium. Early in an acute inflammation response, the circulating neutrophils adhere to the vascular endothelium in response to inflammatory mediators. The adherence of neutrophils to endothelial cells and surfaces is partly due to the expression of cell surface glycoproteins that include the CR3 receptor, lymphocyte function antigen-1 and p150,95 (Stites *et al*., 1991).

Pro-inflammatory cytokines, secreted during inflammation, stimulate the synthesis and expression of E-selectin and Sialyl $Lewis^X$ on the surface of endothelial cells lining the

vaculature in the area of the inflammation. A ligand of Sialyl Lewis^X, L-selectin, is naturally expressed by neutrophils and initiates the attachment of neutrophils to the activated endothelial cells (Figure 7.1) (Nozawa *et al*., 2000). According to Hou *et al.* (2005), CD11b/CD18, also known as CR3, is abundant on activated neutrophils and contributes to neutrophil migration into sites of inflammation. Strong attachment of neutrophils to endothelium and subsequent transmigration is ensured by the activation of integrins.

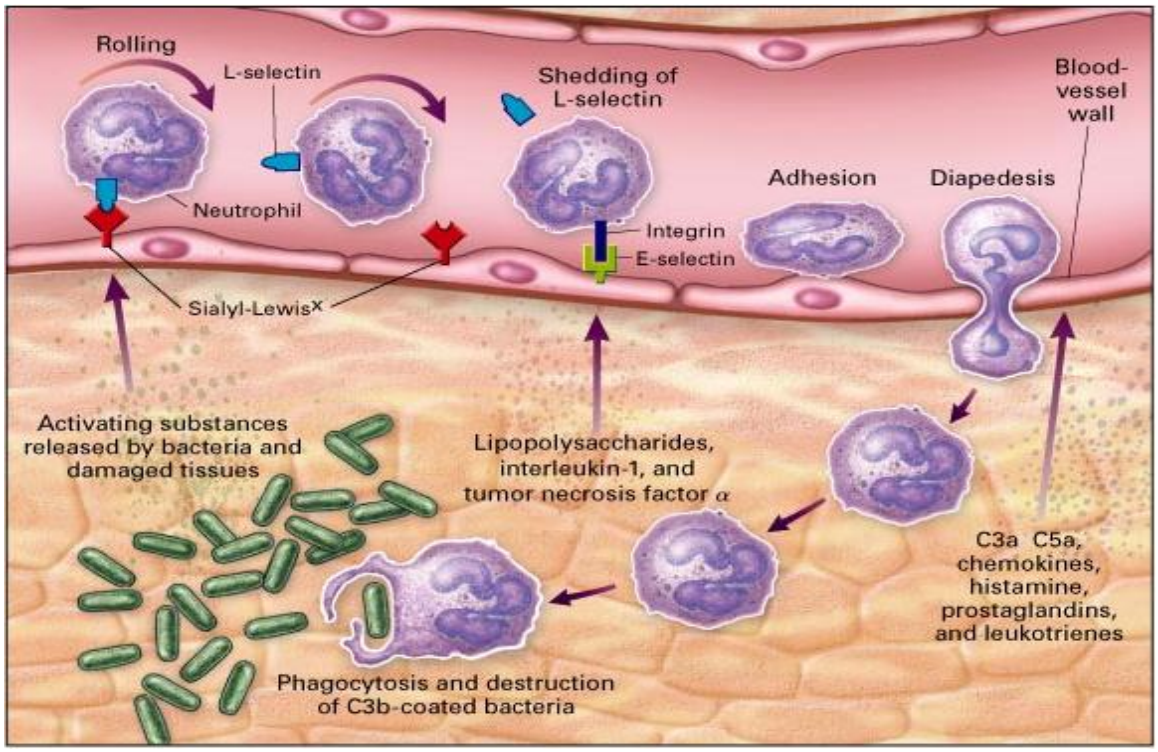


Figure 7.1. A diagrammatic representation of the recruitment, adherence and diapedesis of neutrophils during an inflammatory response (www. biogeonerd.blogspot.com).

Neutrophils are not normally found in tissues unless recruited from the circulation to sites of tissue inflammation. There are a large number of chemotactic factors derived from cellular as well as circulating plasma sources, which recruit neutrophils to sites of tissue inflammation (Stites *et al.*, 1991). The movement of granulocytes from the circulating pool to the peripheral tissues occurs by a process called diapedesis (Figure 7.1). Once in the peripheral tissues, the neutrophils are able to carry out their function of phagocytosis

(Turgeon, 1996). Neutrophils are short-lived cells that phagocytise particles, destroy them and die during the process. The pus formed at the site of infection or injury is mainly composed of dead neutrophils (Nairn *et al*., 2002). Nanomolar concentrations of chemotactic factors released from infected areas have the ability to produce a response from circulating neutrophils that increases their adhesiveness to endothelial cells (Stites *et al*., 1991).

The important stages in the development of an inflammatory response are: the movement of the neutrophils, which may be chemotactic or chemokinetic, the attachment or adhesion to and migration through the vascular endothelium, as well as phagocytosis and digestion of microbes (Figure 7.1). Dysfunction in any of the stages of the neutrophil response would adversely affect the ability of neutrophils to perform their role as a defence mechanism against foreign protein (Hudson *et al*., 1989).

Numerous disease modifying anti-rheumatic drugs have been reported to effect functional responses of neutrophils. Examples of these include: Auranofin (a gold compound) which interferes with chemotaxis *in vitro* and gold sodium thiomalate which alters endothelial E-selectin expression and HUVEC adhesiveness. Several anti-rheumatic drugs may modulate the movement of neutrophils into inflamed areas and thus mediate anti-inflammatory actions (Heimbürger *et al.*, 1998).

Deusch *et al*. (2003) stated that (-)-Δ^9-tetrahydrocannabinol (THC) produced anti-inflammatory effects in animal models during sepsis, acute lung injury and peritonitis. These anti-inflammatory effects were due to the reduction of neutrophil invasion of the tissue and inhibition of neutrophil mediated inflammation. Kraft *et al*. (2004) reported that Δ^9-THC suppressed the antifungal activity of human neutrophils against *Candida albicans* in a dose-dependent manner. These results were confirmed with the use of a synthetic Δ^9-THC-analogue called CP55,940.

In a study done by Smith *et al.* (2001), the anti-inflammatory properties of two non-selective cannabinoid receptor agonists were tested in mouse peritonitis models. It was

found that both HU-210 and WIN55,212-2 blocked the influx of neutrophils into the peritoneal cavity following an intraperitoneal injection of the test compounds. Nilsson *et al*. (2006) found that WIN55,212-2 decreased the transmigration of neutrophils through ECV304 (endothelial cell line spontaneously transformed and established from human umbilical vein) monolayers in response to TNF-α and reduced the release of IL-8 from these cell monolayers. The mechanism of action by which WIN55,212-2 decreases the neutrophil transmigration is still unclear.

Berdyshev *et al*. (1998) conducted an experiment on BALB/c mice and their inflammatory response in the lungs after inhaling LPS. LPS inhalation induced a clear inflammatory response characterized by a massive neutrophil recruitment and TNF-α release. The mice treated with WIN 55,212-2 and Δ^9-THC showed a significant decrease in the LPS-induced neutrophil recruitment.

According to Napimoga *et al*. (2009), male Wistar rats treated with cannabidiol (CBD) presented lower levels of neutrophil infiltration and decreased levels of IL-1β and TNF-α production. A significantly reduced neutrophil accumulation was observed in the CBD-treated group assessed by measuring gingival tissue myeloperoxidase (MPO assay).

Once neutrophils have been recruited to the site of inflammation, local production of several inflammatory mediators is stimulated. These mediators include: cytokines, chemokines, reactive oxygen and nitrogen species, enzymes and metalloproteases that further intensify the inflammatory response and damage surrounding tissue (Napimoga *et al*., 2009). It is thus apparent that the inhibition of the recruitment of neutrophils and therefore decreased cytokine production may be a valuable strategy for treating inflammatory disorders. These facts provide support to determine the possible inhibitory action of Rimonabant on neutrophil attachment to endothelial cells.

7.2. Aim

The aim of this study was to determine the *in vitro* effects of Rimonabant and anandamide alone and in combination on the adhesion of human neutrophils to HUVEC.

7.3. Materials and Methods

The method used in this experiment to isolate human neutrophils from venous blood has previously been described by Ringertz *et al*. (1985). The method used to determine the adhesion of the neutrophils to the HUVEC has previously been described by Håkanson *et al.* (1994). The experiment was repeated 5 times using 5 different blood donors and 5 different umbilical cord donors. Eleven different drug combinations, using Rimonabant at 0.5 µM, 1 µM, 3 µM and anandamide at 5 µM, 10 µM, as well as two control groups were performed using a 96 well plate as summarised in Figure 7.2.

The healthy volunteers used in this study were selected according to inclusion and exclusion criteria to ensure healthy neutrophils for the experiment (refer to Section 2.3). Each patient was required to sign an informed consent form confirming their authorization for the use of their blood during this research project (refer to Annexure C).

7.3.1. Preparation of reagents

All the reagents were sterilised before use and stored in sterile airtight containers. The containers were only opened inside a sterilised laminar flow cabinet (Labotec, model LCA 6000VA) to ensure no contamination of the prepared reagents. HBSS, heparin, 199 medium+ and gelatine were prepared according to the method described in Section 2.3.1. Ammonium chloride, Histopaque, PBS and cell counting fluid were prepared according to the method described in Section 3.3.2. Rimonabant and anandamide were prepared according to the method described in Section 3.3.1. PMA was prepared according to the method described in Section 4.3.1.i.

i. o-Phenylenediamine Solution (Substrate solution)

A. Citrate phosphate buffer:

Citric acid (MW 192.1) 6.724 g/L (Saarchem-Holpro Analytics (Pty) Ltd.)

Na_2HPO_4 (MW 142.0) 9.514 g/L (Saarchem-Holpro Analytics (Pty) Ltd.)

The citrate phosphate buffer was prepared as follows: The two reagents were each dissolved in 400 ml of distilled water. Small amounts of Na_2HPO_4 were added to the citric acid while stirring frequently. The pH of the solution was checked frequently and Na_2HPO_4 was added until the pH of the buffer reached 5 ± 0.5. The solution was then made up to 1000 ml using distilled water.

B. Triton X-100 (0.1%):

One gram of Triton X-100 supplied by Sigma Aldrich (St Louis, USA) was diluted in 1000 ml of distilled water.

C. o-Phenylenediamine (OPD):

One OPD tablet purchased from Sigma Aldrich (St Louis, USA) was diluted in 20 ml of distilled water.

D. H_2O_2:

(Stock solution = 10 molar)

Four hundred microlitres of H_2O_2 (4 µmol) supplied by Sigma Aldrich (St Louis, USA) was diluted in 1000 ml of distilled water.

Solutions **A**, **B**, **C** and **D** were mixed together and then referred to as the OPD substrate solution.

ii. H_2SO_4 ((1 N) Stop solution)

Twenty eight millilitres of H_2SO_4 supplied by Saarchem-Holpro Analytics (Pty) Ltd., Krugersdorp, RSA was slowly added to 800 ml distilled water, made up to 1000 ml and stored at 4^{o}C.

7.3.2. Experimental procedures

i. Isolation and preparation of human neutrophils

This procedure was carried out after the preparation, drug exposure and 24 hour pre-incubation of the HUVEC as described in the next section. Venous blood was collected from a healthy volunteer and heparinised (5 units heparin/ml blood). Human neutrophilic polymorphonuclear leukocytes (PMN) were isolated from the blood using a standard step gradient separation on Histopaque. This was achieved by carefully loading 35 ml of the donor's blood onto 15 ml Histopaque with a Pasteur pipette and then centrifuging the mixture for 25 minutes at 650 *g* (at room temperature). The top plasma layer, as well as the lymphocyte/monocytes layer were removed with a Pasteur pipette and discarded. The granulocyte layer, ± 3 mm on top of the red blood cells, was carefully removed from the tube and placed into a clean sterile 50 ml centrifuge tube and filled with ice-cold ammonium chloride lysing solution. The cell suspension was mixed well and allowed to stand on ice for 10 minutes. This step was done to lyse contaminating red blood cells present in the cell suspension. The solution was centrifuged for 10 minutes at 200 *g* and the supernatant discarded without disturbing the pellet. The ammonium chloride lysing step was repeated if the pellet still contained red blood cells (reddish colour). The pellet (containing granulocytes of which neutrophils are the most abundant) was resuspended in PBS (tube filled with PBS). In the case of several tubes being used, the pellets of all the tubes were pooled before adding PBS. The resuspension (in PBS) step was included to wash off any remaining ammonium chloride. The cell suspension was centrifuged for 10 minutes at 200 *g*. The supernatant was discarded and the pellet was resuspended in cold HBSS. The volume of HBSS added depended on the number of cells in the pellet. If the cell concentration was high, the pellet was diluted further to facilitate counting. E.g. if 250 ml of blood was used, about 8-15 ml of HBSS was added, if 30 ml of blood was used, about 1-3 ml HBSS was added. An aliquot of the cells was counted using cell counting fluid and a glass haemocytometer (as described in Section 3.3.3. iii). The cells were diluted in HBSS to ± 2 x 10^6 cells/ml HBSS. The sealed tubes containing the isolated neutrophils were placed in a 37°C water bath for 15 minutes to reacclimatise and were then used immediately in the experiments and could not be stored for later use.

ii. Neutrophil adhesion assay

Experimental procedure: The HUVEC were removed from a 90% confluent flask using a sterile tissue culture flask scraper (Trypsin affects the cell surface and thus causes damage to the ICAM needed for neutrophil adhesion to the cell surface). The HUVEC were placed into a 15 ml sterile centrifuge tube and the tube was filled with 199 medium+. The cell suspension was centrifuged for 8 minutes at 200 *g*, the supernatant discarded and the pellet resuspended in 1 ml 199 medium+. The cells were counted using cell counting fluid and a haemocytometer (as described in Section 3.3.3. iii) and diluted in 199 medium+ to 1 x 10^5 cells/ml medium. The cell suspension (100 µl), as well as 199 medium+ (40 µl) were placed into each well of a gelatine pre-coated 96 well tissue culture plate (described in Section 2.3.2.ii) and incubated in a 37°C/5% CO_2 incubator for 48 hours. After the incubation period, 20 µl of Rimonabant (various concentrations) and 20 µl of anandamide (various concentrations) was added to the treatment wells and 40 µl of 199 medium+ was added to the control wells (Figure 7.2). The HUVEC were allowed to incubate in a 37°C/5% CO_2 incubator for an hour.

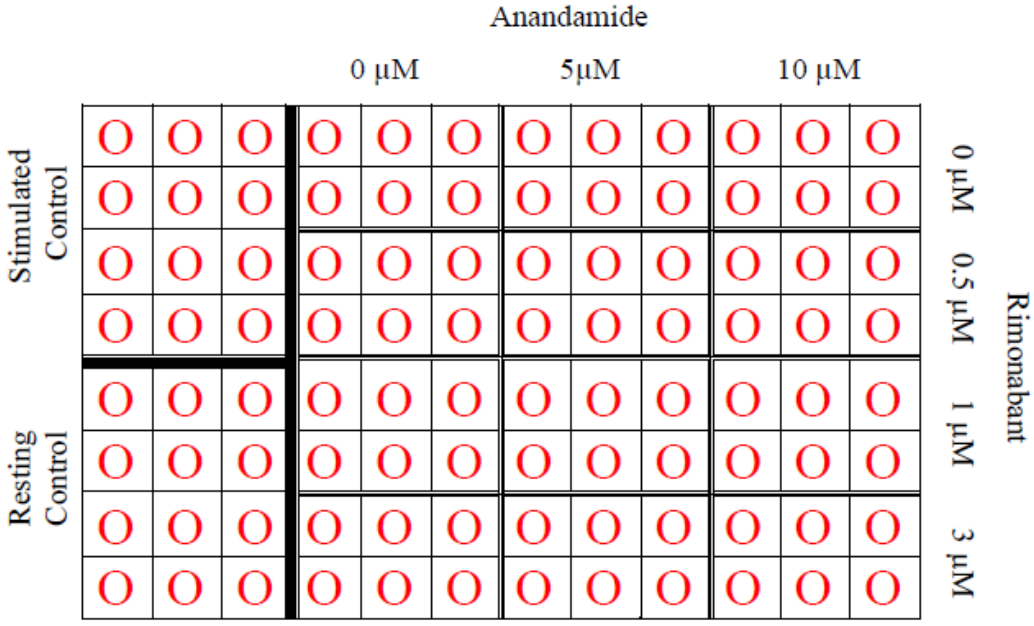


Figure 7.2. A diagrammatic representation of the different drug concentrations and combinations used during this experiment. Three different concentrations of anandamide and four different concentrations of Rimonabant were placed in eleven different combinations, in a 96 well tissue culture plate.

Following the incubation period, the supernatant was removed from each of the 96 wells containing HUVEC. Each well was rinsed with 150 µl HBSS to remove the spent medium. HBSS (160 µl) was added to each well, as well as 20 µl of freshly prepared neutrophil suspension (2.5 x 10^6 cells per well). The freshly prepared PMA solution (20 µl) was added to each of the wells, excluding the resting control wells. HBSS (20 µl) was added to the resting control wells. The tissue culture plates were re-incubated for 1 hour to allow for stimulation and adhesion. The supernatant of each well was removed and HUVEC were rinsed by adding 200 µl HBSS to each well (this rinsing step was repeated 3-4 times) and the remaining supernatant discarded. The OPD substrate solution (100 µl) (refer Section 7.3.1.i) was added to each well and was allowed to stand for 5 minutes at room temperature. The stop solution (100 µl) (refer Section 7.3.1.ii) was added to each well of the tissue culture plate. The absorbance was measured spectrophotometrically on a Universal Microplate Reader (Elx800 UV, Bio-Tek Instruments) using a wavelength of 450 nm.

7.4. Results

As tabulated in Table 7.1, the results are expressed as the mean percentage of untreated controls ± SEM. Each value represents the mean of 5 independent experiments. The P-values compare the experimental values with the controls and were obtained by Kruskal-Wallis test. The results, mean and statistics were obtained using the GraphPad Prism 4 software package.

The results are presented in Table 7.1 and are expressed as the percentage of adherence of neutrophils to stimulated HUVEC compared to the untreated stimulated control values. Table 7.1 illustrates the results obtained from a stimulated control group and eleven different combination concentrations. Twelve groups were defined by the concentrations of the combination of the two compounds used to treat the cells.

Table 7.1. The effects of Rimonabant and anandamide, alone and in combination on the adhesion of PMA stimulated neutrophils to HUVEC. Results expressed as percentage of adherence of neutrophils compared to the relevant untreated stimulated control values.

	Concentrations of AEA (μM)		
	0	**5**	**10**
AC (μM)			
0	100 ± 0.05	94 ± 6.66	96 ± 4.66
0.5	97 ± 5.81	94 ± 6.47	98 ± 3.29
1	97 ± 7.16	92 ± 8.96	99 ± 0.89
3	91 ± 5.05	92 ± 7.02	98 ± 2.83

The results indicate that all treatment with these compounds only very slightly affected the neutrophil adhesion. Anandamide alone had no significant effect on adhesion, whereas Rimonabant used alone at a concentration of 3 μM, inhibited the neutrophil adherence to HUVEC by 9%. Rimonabant in combination with anandamide had a lowering effect on the adhesion of human neutrophils, but the most notable decrease was observed following the treatment with Rimonabant (1 μM) combined with anandamide (5 μM) and Rimonabant (3 μM) combined with anandamide (5 μM) which decreased the neutrophil adhesion by 8%. These results showed no statistical significance. No statistically significant effects were seen for any of the drug concentrations used in this experiment on the adhesion of PMA stimulated human neutrophils to HUVEC.

7.5. Discussion

The method used to determine the effect of Rimonabant and anandamide on the adhesion of human neutrophils to HUVEC, was based on the myeloperoxidase (MPO) activity of the bound cells. This neutrophil assay measured the relative number of PMA stimulated neutrophils that adhere to HUVEC and the number of adhering neutrophils was quantified by analysing MPO release. One of the well-known methods for quantitating granulocytes is by detection of peroxidase in tissue homogenates or extracts, since neutrophils contain substantial amounts of myeloperoxidase (MPO) (Schneider *et al*., 1996). The ability of HUVEC to express ICAM-1 on their surface enhances the capability of neutrophils to adhere to these cells.

Neutrophils play a very important and vital role in the host's defence against microorganisms. Unfortunately though, these effector cells have been implicated in mediating tissue damage linked to chronic inflammatory diseases (Marzocchi-Machado *et al*., 2000). It is not known whether Rimonabant can influence neutrophil adhesion *in vitro* and such information could provide much needed and useful mechanistic data regarding this cannabinoid receptor ligand.

During this assay, neutrophils were stimulated with PMA to facilitate neutrophil adhesion followed by the removal of unbound neutrophils with a wash step. The bound neutrophils were lysed and quantitated with a colour developing substrate solution and the highly coloured product then quantitated spectrophotometrically. This method has previously been described by Byrne *et al*. (2002).

As observed from the results obtained during this experiment, there was no significant difference with relation to the neutrophil adhesion to HUVEC following the treatment with various combination concentrations of Rimonabant and anandamide, and also no significant effect following treatment with either test compound individually. According to the results of an *in vivo* study conducted on C57BL/6 female mice, the activation of CB_2 receptors attenuates microvascular leukocyte/endothelial interactions, whereas the activation of CB_1 receptors had no significant effect on this interaction (Ni *et al*., 2004). Ni *et al*. (2004) concluded that the specific receptor involved in the inhibition of leukocyte adhesion in encephalomyelitis induced mice, was the CB_2 receptor. This correlates with the results found during a study done on human liver sinusoidal endothelial cells (HLSECs) (Rajesh *et al.,* 2007). The Rajesh *et al.* (2007) publication indicated that the CB_2 receptor agonist, HU-308, markedly inhibited PMN adhesion to HLSECs and this effect was attenuated by CB_2 antagonists. These finding might explain the lack of inhibitory effect observed from the CB_1 receptor antagonist (Rimonabant) and the CB_1 receptor agonist (anandamide) during this study.

7.6. Conclusion

Considering the central role that neutrophils play in inflammatory diseases and previous data supporting the theory that cannabinoid ligands are involved in neutrophil adhesion, studying the effects of Rimonabant on the adhesion of human neutrophils was logical. The results of this adhesion experiment however, provide no significant evidence to support the hypothesis that Rimonabant or anandamide (alone and in combination) inhibits the adhesion of PMA stimulated human neutrophils to HUVEC which exhibit high expression of CR3 adhesion molecules on their surfaces. The results of this study have further strengthened the data currently available, indicating that the CB_1 receptor is not involved in the inhibition of neutrophil adhesion. The cannabinoid-mediated mechanism involved in the inhibition of neutrophil adhesion has been claimed to be the CB_2 receptor in other study models and the findings from this present study indicates that this effect is applicable to *in vitro* adhesion effects of neutrophils to HUVEC. Further studies should be conducted to verify the inhibitory action of a CB_2 ligand in this current study model.

The conclusion of this study is that even though there is a possibility that cannabinoids decrease the adhesion of neutrophils, this effect is not due to CB_1 receptor ligands and thus the mechanism of action by which Rimonabant exerts an anti-inflammatory action does not appear to involve the adhesion of neutrophils to the endothelium.

Chapter 8: Discussion and conclusion

8.1. Discussion

Proliferation, activation, expression of adhesion molecules and differentiation into effector cells are important responses of the cells of the immune system to an invading pathogen. This immune response is essential to protect the host against bacteria or viruses and to maintain a healthy body. However, persistent activation of the cells of the immune system may lead to chronic inflammatory diseases which are harmful and destructive to the host tissue. Development of a safe anti-inflammatory drug for chronic treatment could thus relieve, reduce or even cure millions of patients suffering from numerous chronic inflammatory diseases.

The discovery of the cannabinoid receptors i.e. CB_1 and CB_2, the isolation and synthesis of endogenous ligands such as anandamide as well as the development of novel ligands including Rimonabant, triggered scientists' interest in cannabinoid pharmacology. Rimonabant, classified as a CB_1 antagonist, is also referred to as a CB_1 inverse agonist due to its well documented inverse cannabimimetic properties. Rimonabant's behavioural and biochemical effects are therefore opposite in direction to the effects produced by Δ^9-THC. Rimonabant was developed for weight reduction and as a pharmacological aid for smoking cessation, but in addition several studies indicate that this CB_1 inverse agonist possesses potential anti-inflammatory properties (Bergman *et al*., 2008). The aim of this study was to investigate the possible anti-inflammatory properties of Rimonabant on primary HUVEC cultures, on human macrophages and human neutrophils *in vitro*.

During this study, the methods for isolation and culturing primary human umbilical vein endothelial cells (HUVEC) were adapted and developed to allow the consistent production of healthy, viable and uncontaminated primary HUVEC cultures for future use. This cell type was selected for this study due to the abundant evidence indicating that endothelial cells produce several important molecules vital to the inflammatory

response and the confirmation that CB_1 receptor mRNA is present in endothelial cells (Liu *et al.*, 2000). HUVEC is a cell type commonly used for the efficacy assessment during drug development (Asahina *et al*., 2000; Nizamutdinova *et al*., 2007; Ou *et al*., 2006).

Following a cytotoxicity assessment, Rimonabant as well as anandamide proved to have a dose related cytotoxic effect on both HUVEC and human lymphocytes. As lymphocytes and endothelial cells are partially responsible for the release of pro-inflammatory cytokines, they play a crucial role in inflammation (Holtman *et al*, 1995). The results of the cytotoxicity study indicated that Rimonabant initiated a cytotoxic effect from a concentration of 6.25 µM and anandamide at concentration of greater than 25 µM. The possibility that the cytotoxic effects observed were due to the ethanol used to dissolve the experimental compounds, were excluded. These results supported the use of lower concentrations of these drugs to eliminate cytotoxicity during further experimentation. Nakajima *et al* (2006) obtained promising results using anandamide at concentrations such as 1 µM, 5 µM and 10 µM and combined anandamide with CB_1 specific antagonists (AM251 or SR 144528) at a concentration of 1 µM each. These results showed potential anti-inflammatory effects and these combinations of concentrations were used as a guideline for the experiments in this study.

After adapting the isolation and culturing methods for the HUVEC cells and determining the non-toxic *in vitro* concentrations of the experimental compounds, the possible anti-inflammatory properties could be investigated. Several major inflammatory pathways exist including the arachidonic acid/ COX pathway and the NF-κB pathway. The COX pathway is a complex biochemical pathway that leads to the production of prostaglandins and thromboxanes whose initial effect is pain and tissue destruction, followed by healing and recovery. The NF-κB pathway on the other hand, acts as a switch that turns inflammation on and off in the body. NF-κB can turn on the particular genes that lead to the production of pro-inflammatory cytokines, protein kinases that regulate the expression of other target genes necessary for maintaining the inflammatory state, various adhesion molecules as well as chemokines (Maroon *et al*., 2006). The NF-κB pathway

was selected for this research project as a starting point in elucidating the probable mechanism involved in Rimonabant's anti-inflammatory properties. The identification of a product that is able to inhibit any component of the NF-κB pathway could have profound implications for therapeutic manipulation of regulatory circuits controlling the inflammatory process, regardless of its causes.

The major adhesion-promoting receptor on the cell surface of activated phagocytes is CR3 and it has been documented that agents that block this surface molecule are beneficial in the treatment of chronic inflammation by inhibiting the recruitment of leukocytes into tissues (Bansal *et al*., 2003). However, in this study it was found that Rimonabant and anandamide had no significant effect on the expression of this molecule by human neutrophils.

A BD Cytometric Bead Array ready-to-use human inflammation kit was used to evaluate the effect of the test compounds on the production of cytokines by HUVEC and human macrophages. The results confirmed previous finding indicating that anandamide possesses anti-inflammatory properties by inhibiting the production of select cytokines. Anandamide inhibited the production of all the detectable cytokines during this experiment (IL-8, IL-1β and IL-6 in both cell types and IL-10 and TNF-α in macrophages). One of the most interesting finding presented in the results was the dose-response relationship of AC. The response was characteristic of a bell-shaped dose-response curve, suggesting that low and high concentrations of Rimonabant produced anti-inflammatory responses whereas mid-range concentrations produced pro-inflammatory responses. The pro-inflammatory responses illustrated in the results following treatment with 1 μM of Rimonabant can be ascribed to an inverse cannabimimetic effect of Rimonabant, whereas the mechanism by which anti-inflammatory effects are produced is still somewhat unclear. This unusual finding might be the result of involvement of a different receptor, maybe even CB_2. Rimonabant is known to be a highly selective CB_1 antagonist/inverse agonist, however the possibility exists that in a CB_1 saturated state, Rimonabant could bind to CB_2 and activates the anti-

inflammatory response associated with this cannabinoid receptor. This scenario would explain the anti-inflammatory effect of Rimonabant observed at high concentrations.

Adhesion of certain cells (including neutrophils) to endothelial cells is crucial in the development of inflammation and became the focus of the next part of the research project. Neutrophils adhere to the endothelial cells through the binding to specific expressed ICAM molecules. Inhibiting ICAM-1 expression by the endothelial cells could potentially reduce inflammation and a drug possessing this ability would be of great therapeutic interest. A flow cytometer based assay was used to determine the expression of ICAM-1 on the surface of HUVEC. The results obtained during this study indicated negligible levels of ICAM-1 expression by HUVEC during resting and only a doubling in concentration after IL-1β stimulation. These findings contradict previous results obtained by Wheller *et al*. (1997) and several critical differences in the experimental methods used during each of the two studies were identified as possible reasons, which could have resulted in the discrepancies observed. These experimental differences include the concentration of cells used during the experiment, the concentration of stimulant the cells were exposed to as well as the number of events counted during the flow-cytometer assay. No significant inhibitory action of Rimonabant or anandamide (alone and in combination) with respect to the expression of ICAM-1 on resting or IL-1β stimulated HUVEC were observed during this study using the current method. Bearing in mind the atypical level of ICAM-1 expressed during this experiment and the possible enhancement steps pinpointed to improve the current method, this experiment should be revisited to be able to provide a feasible scientific evaluation.

A neutrophil assay was conducted to measure the number of PMA stimulated neutrophils that adhered to HUVEC. The results obtained indicated that Rimonabant alone or in combination with anandamide had no significant effect on the adhesion of neutrophils to HUVEC. These findings support the results published by Ni *et al*. (2004) and Rajesh *et al.* (2007) suggesting that the specific receptor involved in the inhibition of leukocyte adhesion to endothelial cells is CB_2 and not CB_1 receptors. This explains the lack of inhibitory effect observed from the CB_1 receptor antagonist Rimonabant (AC) and the

CB_1 receptor agonist anandamide (AEA) during this study. The slight inhibitory effects presented in the results were only seen at high concentrations of Rimonabant. As much as a 9% decrease in adhesion was observed following pre-treatment of HUVEC with 3 µM of Rimonabant. This links to the anti-inflammatory effects seen during the cytokine assay and strengthens the theory that although Rimonabant has a strong affinity for the CB_1 receptor, a low secondary affinity toward CB_2 may be possible. If CB_2 is the receptor influencing neutrophils adhesion and inhibition thereof is only observed at high concentrations of Rimonabant, one can conclude that Rimonabant could bind to CB_2 once the CB_1 receptors are saturated.

8.2. Conclusion

The main aim of this study was to investigate the possible *in vitro* anti-inflammatory effects of Rimonabant (AC) alone and in combination with anandamide (AEA) on HUVEC and macrophage cell cultures. All the objectives of this study were addressed with the exception of ICAM-1 expression. Reassessment of the research technique following the uncharacteristic results obtained, highlight possible improvements to the ICAM-1 expression method and thus additional experiments would be necessary in order to produce conclusive results.

The CR3 expression was evaluated and experimental results facilitated the elimination of the possible manipulation of CR3 expression as a mechanism of action for both Rimonabant and AEA. The experimental compounds had no significant effect on the expression of CR3 by human neutrophils. Knowing the crucial role that cytokines play during an inflammatory response, studying Rimonabant's effect on the production of these proteins was informative. The results proposed that one of the mechanisms by which Rimonabant inhibits inflammatory reactions is by acting on the cytokine producing cells. As suggested by the bell-shaped dose-response curve, Rimonabant causes pro- and anti- inflammatory responses depending on the concentration available in the cell culture media. Anandamide proved to have anti-inflammatory properties by inhibiting the production of cytokines by HUVEC and human macrophages. This effect by anandamide was however attenuated by AC. At a concentration of 1 µM Rimonabant

completely displaces anandamide, blocks the anti-inflammatory effect of the agonist and fully functions as an inverse-agonist at the CB_1 site. Increasing the concentration of Rimonabant results in a completely different effect. Cytokine production decreases to below the basal level at a Rimonabant concentration of 3 µM, suggesting either inverse agonist activity or involvement of a different receptor. It is possible that during a completely CB_1 saturated state, Rimonabant binds to CB_2 receptors and activates the anti-inflammatory response associated with this cannabinoid receptor. Although no statistically significant results were obtained during the neutrophil adhesion assay, the results mimic the anti-inflammatory properties of Rimonabant at higher concentrations. Although a specific mechanism of action for Rimonabant could not be uncovered there is evidence that several possible mechanisms can be excluded.

The results obtained during this study, supports observations made by other researchers and the hypothesis that Rimonabant has anti-inflammatory effects. The results provide motivation for further experimentation to better understand these anti-inflammatory actions of Rimonabant.

References

Akihiko A, Tada Y, Nakamura K, Tamaki K. Griseofulvin has a potential to modulate the expression of cell adhesion molecules on leukocytes and vascular endothelial cells. International Immunopharmacology, 2001; 1: 75-83.

Ao X, Wang X, Lennartz MR, Loegering DJ, Stenken JA. Multiplexed cytokine detection in microliter microdialysis samples obtained from activated cultured macrophages. Journal of Pharmaceutical and Biomedical Analysis, 2006; 40: 915-921.

Austyn JM, Wood KJ. Principles of cellular and molecular immunology. Oxford University Press, 1993.

Bansal VS, Vaidya S, Somers EP, Kanuga M, Shevell D, Weikel R, Detmers PA. Small molecule antagonists of complement receptor type 3 block adhesion and adhesion-independent oxidative burst in human polymorphonuclear leukocytes. Journal of Pharmacology and Experimental Therapeutics, 2003: 1016-1024.

Barltrop JA, Owen TC, Cory AH, Cory JG. 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl) tetrazolium, inner salt (MTS) and related analogs of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reducing to purple water-soluble formazans as cell-viability indicators. Bioorganic & Medicinal Chemistry Letters, 1991; 1: 611-614.

Barnes PJ, Karin M. Nuclear Factor-κB – A privotal transcription factor in chronic inflammatory diseases. The New England Journal of Medicine, 1997; 336: 1066-1071.

Begg M, Pacher P, Bátkai S, Osei-Hyiaman D, Offertáler L, Mo FM, Liu J, Kunos G. Evidence for novel cannabinoid receptors. Pharmacology & Therapeutics, 2005; 106: 133-145.

Berdyshev E, Boichot E, Corbel M, Germain N, Lagente V. Effects of cannabinoid receptor ligands on LPS-induced pulmonary inflammation in mice. Pharmacology Letters, 1998; 63: 125-129.

Bergman J, Delatte MS, Paronis CA, Vemuri K, Thakur GA, Makriyannis A. Some effects of CB_1 antagonist with inverse agonist and neutral biochemical properties. Physiology & Behavior, 2008; 93: 666-670.

Brown Z, Gerritsen ME, Carley WW, Streier RM, Kunkel SL, West-wick J. Chemokines gene expression and secretion by cytokine activated human micro vascular endothelial cells. Differential regulation of monocytes chemoattractant protein-1 and interleukins-8 in response to interferon-γ. Journal of Pathology, 1994; 145: 913-921.

Burstein SH. The cannabinoid acids: Nonpsychoactive derivatives with therapeutic potential. Pharmacology & Therapeutics, 1999; 82: 87-96.

Byrne MF, Corcoran PA, Atherton JC, Sheehan DM, Murray FE, Fitzgerald DJ, Murphy JF. Stimulation of adhesion molecule expression by *Helicobacter pylori* and increased neutrophil adhesion to human umbilical endothelial cells. FEBS Letters, 2002; 532: 411-414.

Calandra B, Portier M, Kernéis A, Delpech M, Carillon C, Le Fur G, Ferrara P, Shire D. Dual intracellular signalling pathways mediated by the human cannabinoid CB_1 receptor. European Journal of Pharmacology, 1999; 374: 445-455.

Calignano A, La Rana G, Giuffrida A, Piomelli D. Control of pain initiation by endogenous cannabinoids. Nature, 1998; 394: 277-281.

Cannabinoid CB_2 receptor.
www.cnsforum.com/imagebank/section/substance_abuse/default.aspx

Clayton N, Marhall FH, Bountra C, O'Shaughnessy CT. CB1 and BC2 cannabinoid receptors are implicated in inflammatory pain. Pain, 2002; 96: 253-260.

Collins RG, Huang YF, Guevara NV, Hicks MJ, Chan L, Beaudet AL. P-selectin or intercellular adhesion molecule (ICAM)-1 deficiency substantially protects against atherosclerosis apolipoprotein E-deficient mice. Journal of Experimental Medicine, 2000; 191: 189-283.

Croci T, Landi M, Galzin AM, Marini P. Role of cannabinoid CB_1 receptors and tumor necorsis factor-alpha in the gut and systemic anti-inflammatory activity of SR 141716 (Rimonabant) in rodents. British Journal of Pharmacology, 2003; 140: 115-122.

Cytokines. www.new-science-press.com/info/illustration_files/nsp-immunity-biomed.brown.edu

De Petrocellis L, Melek D, Palmisano A, Bisogno T, Laezza C, Bifulco M, Di Marzo V. The endogenous cannabinoid anandamide inhibits human breast cancer cell proliferation. Proceedings of the National Academy of Sciences of the USA, 1998; 95: 8375-8380.

Deusch E, Kraft B, Nahlik G, Weigl L, Hohenegger M, Kress HG. No evidence for direct modulatory effects of Δ^9-Tetrahydrocannabinol on human polymorphonuclear leukocytes. Journal of Neuroimmunology, 2003; 141: 99-103.

DiMarzo V, DePetrocellis L, Fessa F, Ligreste A, Bisogno T. Anandamide receptors. Prostaglandins, Leukotriens and Essential Fatty Acids, 2002; 66: 377-391.

Egerton A, Allison C, Brett RR, Pratt JA. Cannabinoids and prefrontal cortical function: Insights from Preclinical studies. Neuroscience and Biobehavioral Reviews, 2006; 30: 680-695.

Flygare J, Gustafsson K, Kimby E, Christensson B, Sander B. Cannabinoid receptor ligands mediate growth inhibition and cell death in mantle cell lymphoma. Federation of European Biochemical Societies Letters, 2005; 579: 6885-6889.

Fox S. Human physiology, Fourth Edition. W.C. Brown (1993) ASIN: B001KT69XM Product Dimensions, 1993.

Gallily R, Breuer A, Mechoulam R. 2-Arachidonylglycerol, an endogenous cannabinoid, inhibits tumor necrosis factor-a production in murine macrophages, and in mice. European Journal of Pharmacology, 2000; 406: R5-R7.

Goncharova LB, Tarakanov AO. Molecular networks of brain and immunity. Brain Research Reviews, 2007; 55: 155-166.

Graham IL, Gresham HD, Brown EJ. An immobile subset of plasma membrane CD11b/CD18 (Mac-1) is involved in phagocytosis of targets recognized by multiple receptors. Journal of Immunology, 1989; 142: 2352-2358.

Håkanson L., Nielson L. S., & Teder P. Measurement of neutrophil and eosinophil adhesion to E-selectin, VCAM-1 and ICAM-1 by the use of transfected fibroblast cell lines. Journal of Immunolgical Methods, 1994; 176: 53-66.

Heimbürger M, Lerner R, Palmblad J. Effects of antirheumatic drugs on adhesiveness of endothelial cells and neutrophils. Biochemical Pharmacology, 1998; 56: 1661-1669.

Herzenberg LA, Parks D, Sahaf B, Perez O, Roederer M, Herzenberg LA. The history and future of the fluorescence activated cell sorter and flow cytometry: A view from Stanford. Clinical Chemistry, 2002; 48: 1819-1827.

Hoang-Ngoc M, Gebrane-Younes J, Smadja A, Orcel L. Structure and function of the foetal umbilical cord. European Journal of Obstetrics & Gynaecology and Reproductive Biology, 1985; 14: 973-9.

Hou Y, Hsu C, Yeh C, Chiu W, Pai M, Yeh S. Effects of glutamine on adhesion molecule expression and leukocyte transmigration in endothelial cells exposed to arsenic. Journal of Nutritional Biochemistry, 2005; 16: 700-7004.

Hudson L, Hay FC. Practical immunology, Third Edition. Blackwell Science Ltd a Blackwell Publishing Company, 1989.

Hynes J, Hill R, Papkovsky DB. The use of a fluorescence-based oxygen uptake assay in the analysis of cytotoxicity. Toxicology in Vitro, 2006; 20: 785-792.

Ihenetu D, Molleman A, Parsons ME, Whelan CJ. Inhibition of inteleukin-8 release in the human colonic epithelial cell line HT-29 by cannabinoids. European Journal of Pharmacology, 2003; 458: 207-215.

Ihenetu D, Molleman A, Parsons ME, Whelan CJ. Pharmacological characterisation of cannabinoid receptors inhibiting interleukins 2 release from human peripheral blood mononuclear cells. European Journal of Pharmacology, 2003; 464: 207-215.

Imaizumi T, Itaya H, Fujita K, Kudoh D, Kudoh S, Mori K, Fujimoto K, Matsumiya T, Yoshida H, Satoh K. Expression of tumor necrosis factor-α in cultured human endothelial cells stimulated with lipopolysaccharide or interleukins-1α. Arteriosclerosis, Thrombosis, and Vascular Biology, 2000; 20: 410-415.

Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of Human Endothelial Cells Derived from Umbilical Veins. The Journal of Clinical Investigation, 1973; 52: 2745-2756.

Janeway CA, Travers P, Walport M, Sholomchik M. Immunobiology: the immune system in health and disease, 6th Edition. New York: Garland Science, 2005.

Kadl A, Leitinger N. The role of endothelial cells in the resolution of acute inflammation. Antioxidants & Redox Signaling, 2005; 7: 1744-1754.

Kaminski NE. Inhibition of the cAMP signalling cascade via cannabinoid receptors: a putative mechanism of immune modulation by cannabinoid compounds. Toxicology Letters, 1998; 102-103: 59-63.

Kikuchi H, Isshi K, Hirohata S. Inhibitory effects of bucillamine on the expression of vascular cell adhesion molecule-1 in human umbilical vein endothelial cells. International Immunopharmacology, 2004; 4: 119-126.

Kim HG, Kim JY, Hwang YP, Lee KJ, Lee KY, Kim DH, Kim DH, Jeong HG. The coffee diterpene kahweol inhibits tumor necrosis factor-α-induced expression of cell adhesion molecules in human endothelial cells. Toxicology and Applied Pharmacology, 2006; 217: 332-341.

Kiszel P, Makó V, Prohászka Z, Cervenak L. Interleukin-6-174 promoter polymorphism does not influence IL-6 production after LPS and IL-1β stimulation in human umbilical cord vein endothelial cells. Cytokine, 2007; 40: 17-22.

Klein TW, Friedman H, Specter S. Marijuana, immunity and infection. Journal of Neuroimmunology, 1998; 83: 102-115.

Klein TW, Lane B, Newton CA, Friedman H. The cannabinoid system and cytokine network. Society for Experimental Biology and Medicine, 2000; 225: 1-8.

Kliman HJ. The umbilical cord (from the encyclopaedia of reproduction). Yale University School of Medicine, 2005. http://www.med.yale.edu/obgyn/kliman/placenta/articles/EOR_UC/Umbilical_Cord.html

Konkle BA, Ginsburg D. The addition of endothelial cell growth factor and heparin to human umbilical vein endothelial cell cultures decreases plasminogen activator inhibitor-1 expression. Journal of Clinical Investigation, 1988; 82: 579-585.

Kraft B, Wintersberger W, Kress HG. Cannabinoid receptor-independent suppression of the superoxide generation of human neutrophils (PMN) by CP55 940, but not by anandamide. Life Sciences, 2004; 75: 969-977.

Liu J, Gao B, Mirahahi F, Sayal AJ, Khanolkar AD, Makriyannis A, Kunos G. Functional CB1 cannabinoid receptors in human vascular endothelial cells. Biochemistry Journal, 2000; 346: 835-840.

Lu T, Newton C, Perkins I, Friedman H, Klein TW. Role of cannabinoid receptors in Delta-9-tetrahydrocannabinol suppression of IL-12p40 in mouse bone marrow-derived dendritic cells infected with Legionella pneumophila. European Journal of Pharmacology, 2006; 532: 170-177.

Male D, Brostoff J, Roth DB, Roitt I. Immunology, Seventh Edition. Elsevier, 2006.

Maroon JC, Bost JW, Borden MK, Lorenz KM, Ross NA. Natural anti-inflammatory agents for pain relief in athletes: inflammatory pathways. Neurosurgery Focus, 2006; 21.

Marzocchi-Machado CM, Russo EMS, Alves CMOS, Polizello ACM, Azzolini AECS, Lucisano-Valim YM. Effect of low-dose prednisone in vivo on the ability of complement receptor to mediate an oxidative burst in rat neutrophils. Immunopharmacology, 2000; 49: 247-254.

McKallip RJ, Lombard C, Fisher M, Martin BR, Ryu S, Grant S, Nagarkatti PS, Nagarkatti M. Targeting CB_2 cannabinoid receptors as a novel therapy to treat malignant lymphoblastic disease. Blood, 2002; 100: 627-634.

Meager A. Cytokine regulation of cellular adhesion molecule expression in inflammation. Cytokine and Growth Factor Reviews, 1999; 10: 27-39.

Mestre L, Correa F, Docagne F, Clemente D, Guaza C. The synthetic cannabinoid WIN 55,212-2 increases COX-2 expression and PGE2 release in murine brain-derived endothelial cells following Theiler's virus infection. Biochemical Pharmacology, 2006; 72: 869-880.

Mestre L, Docagne F, Correa F, Loría F, Hernangómez M, Borrell J, Guaza C. A cannabinoid agonist interferes with the progression of a chronic model of multiple sclerosis by down regulating adhesion molecules. Molecular and Cellular Neurosciences, 2009; 40: 258-266.

Miyamoto H, Sugawara I, Azuma A, Saito Y, Kohno N, Kudoh S. Differential secretion of cytokines and adhesion molecules by HUVEC stimulated with low concentrations of bleomycin. Cellular Immunology, 2002; 219: 73-81.

Molinari BL, Tasat DR, Palmieri MA, Cabrini RL. Kinetics of MTT-formazan exoccytosis in phagocytic and non-phagocytic cells. Micron, 2005; 36: 177-183.

Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods, 1983; 65: 55-63.

Muller M. Biology of cells and organisms, Third Edition. C. Brown Publishers, 1998.

Nairn R, Helbert M. Immunology for medical students. Mosby International, 2002.

Nakajima Y, Furuichi Y, Biswas KK, Hashiguchi T, Kawahara K, Yamaji K, Uchimura T, Izumi Y, Maruyama I. Endocannabinoid, anandamide in gingival tissue regulates the periodontal inflammation through NF-κB pathway inhibition. FEBS Letters, 2006; 580: 613-619.

Napimoga MH, Benatti BB, Lima FO, Alves PM, Campos AC, Pena-dos-Santos DR, Severino FP, Cunha FQ, Guimaraes FS. Cannabidiol decreases bone resoption by inhibiting RANK/RANKL expression and pro-inflammatory cytokines during experimental periodontitis in rats. International Immunopharmacology, 2009; 9: 216-222.

Ni X, Geller EB, Eppihimer MJ, Eisenstein TK, Adler MW, Tuma RF. Win 55212-2, a cannabinoid receptor agonist, attenuates leukocyte/endothelial interactions in an experimental autoimmune encephalo myelitis model. Multiple Sclerosis, 2004; 10: 158-164.

Nicholson GC, Tennant RC, Carpenter DC, Sarau HM, Kon OM, Barnes PJ, Salmon M, Vessey RS, Tal-Singer R, Hansel TT. A novel flow cytometric assay of human whole blood neutrophil and monocytes CD11b levels: Upregulation by chemokines is related to receptor expression, comparison with neutrophil shape change and effects of a chemokines receptor (CXCR2) antagonist. Pulmonary Pharmacology & Therapeutics, 2007; 20: 52-59.

Nilsson O, Fowler CJ, Jacobsson SOP. The cannabinoid agonist WIN55,212-2 inhibits TNF-α-induced neutrophil transmigration across ECV304 cells. European Journal of Pharmacology, 2006; 547: 165-173.

Nizamutdinova ET, Oh HM, Min YN, Park SH, Lee MJ, Dim JS, Yean MH, Kang SS, Kim SH, Chang KC, Kim HJ. Paeonol suppresses intercellular adhesion molecule-1 expression in tumor necrosis factor-α-stimulated human umbilical vein endothelial cells

by blocking p38, ERK and nuclear factor-κB signalling pathways. International Immunopharmacology, 2007; 7: 343-350.

Nozawa F, Hirota M, Okabe A, Shibata M, Iwamura T, Haga Y, Ogawa M. Elastase activity enhances the adhesion of neutrophil and cancer cells to vascular endothelial cells. Journal of Surgical Research, 2000; 94: 153-158.

Onaivi ES, Chaudhuri G, Abaci AS, Parker M, Manier DH, Martin PR, Hubbard JR. Expression of cannabinoid receptors and their gene transcripts in human blood cells. Neuro-Psychopharmacology and Biological Psychiatry, 1999; 23: 1063-1077.

Ormerod MG. Flow cytometry – A basic introduction. Privately Published, 2008.

Ou HC, Chou FP, Lin TM, Yang CH, Sheu WHH. Protective effects of eugenol against oxidized LDL-induced cytotoxicity and adhesion molecule expression in endothelial cells. Food and Chemical Toxicology, 2006; 44: 1485-1495.

Patrini G, Sacerdote P, Fuzio D, Manfredi B, Parolaro D. Regulation of immune functions in rat splenocytes after acute and chronic in vivo treatment with CP-55,940, a synthetic cannabinoid compound. Journal of Neuroimmunology, 1997; 80: 143-148.

Pertwee RG. Inverse agonism and neutral antagonism at cannabinoid CB_1 receptors. Life Sciences, 2005; 76: 1307-1324.

Pertwee RG, Ross RA. Cannabinoid receptors and their ligands. Prostaglandins, Leukotrienes and Essential Fatty Asids, 2002; 66: 101-121.

Phagocytosis. www.newscentre.lbl.gov/news-releases/2008/11/05/proteomics-study-yields-clues-as-to-how-tuberculosis-might-be-thwarting-the-immune-system/

Rajesh M, Pan H, Mukhopadhyay P, Bátkai S, Osei-Hyiaman, Haskó G, Liaudet L, Gao B, Pacher P. Cannabinoid-2 receptor agonist HU-308 protects against hepatic ischemia/reperfusion injury by attenuating oxidative stress, inflammatory response and apoptosis. Journal of Leukocyte Biology, 2007; 82: 1382-1388.

Read MA, Neish AS, Luscinskas FW, Palombella VJ, Maniatis T, Collins T. The proteasome pathway is required for cytokine-induced endothelial-leukocyte adhesion molecule expression. Immunity, 1995; 2: 493-506.

Ringertz B, Palmblad J, Lindgren JA. Stimulus specific neutrophil aggregation-evaluation of possible mechanism for the stimulus-response apparatus. Journal of Laboratory Clinical Medication, 1985; 106: 132-140.

Rothe A, Rubbert A. Recombinant proteins in rheumatology – recent advances. New Biotechnology, 2011; 28: 502-510.

Roitt I. Essential immunology, Eighth Edition. Wiley-Blackwell, 1994.

Sacerdote P, Massi P, Panerai AE, Parolaro D. In vivo and in vitro treatment with the synthetic cannabinoid CP55,940 decreases the in vitro migration of macrophages in the rat: involvement of both CB_1 and CB_2 receptors. Journal of Neuroimmunology, 2000; 109: 155-163.

Sadeghi MM, Collinge M, Pardi R, Bender JR. Simvastatin modulates cytokine-mediated endothelial cell adhesion molecule induction: involvement of an inhibitory G protein. Journal of Immunology, 2000; 165: 2712-2718.

Sarfaraz S, Afaq F, Adhami V, Mukhtar H. Cannabinoid receptor as a novel target for the treatment of prostate cancer. Cancer Research, 2005; 65: 1635-1641.

Schley M, Ständer S, Kerner J, Vajkoczy P, Schüpfer G, Dusch M, Schmelz M, Konrad C. Predominant CB_2 receptor expression in endothelial cells of glioblastoma in humans. Brain Research Bulletin, 2009; 79: 333-340.

Schneider T, Issekutz AC. Quantitation of eosinophils and neutrophil infiltration into rat lung by specific assays for eosinophils peroxidase and myeloperoxidase. Journal of Immunological Methods, 1996; 198: 1-14.

Schottelius AJG, Baldwin Jr AS. A role for transcription factor NF-κB in intestinal inflammation. International Journal for Colorectal Diseases, 1999; 14: 18-28.

Shalekoff S, Page-Shipp L, Tiemessen CT. Effects of anticoagulants and temperature on expression of activation markers CD11b and HLA-DR on human leukocytes. Clinical and Vaccine Immunology, 1998; 5 (5): 695-702.

Smith SR, Denhardt G, Terminelli C. The anti-inflammatory activities of cannabinoid receptor ligands in mouse peritonitis models. European Journal of Pharmacology, 2001; 432: 107-119.

Smith SR, Terminelli C, Denhardt G. Modulation of cytokine responses in Corynebacterium parvum-primed endotoxemic mice by centrally administered cannabinoid ligands. European Journal of Pharmacology, 2001; 425: 73-83.

Spoto B, Fezza F, Parlongo G, Battista N, Sgro E, Gasperi V, Zoccali C, Maccarrone M. Human adipose tissue binds and metabolises the endocannabinoids anandamide and 2-arachidonoylglycerol. Biochimie, 2006; 88: 1889-1897.

Sommers DK. Sommers' Pharmacology, First English Edition. Pretoria: Sommers, 2002.

Srivastava MD, Srivastava BIS, Brouhard B. Δ^9 Tetrahydrocannabinol and cannabidiol alter cytokine production by human immune cells. Immunopharmacology, 1998; 40: 179-185.

Stannard AK, Riddell DR, Bradley NJ, Hassall DG, Graham A, Owen JS. Apolipoprotein E and regulation of cytokine-induced cell adhesion molecule expression in endothelial cells. Atherosclerosis, 1998; 139: 57-64.

Stites DP, Terr AI. Basic and Clinical Immunology, Seventh Edition. Appleton & Lange, 1991.

Svíženská I, Dubový P, Šulcová A. Cannabinoid receptors 1 and 2 (CB_1 and CB_2), their distribution, ligands and functional involvement in nervous system structures – A short review. Pharmacology, Biochemistry and Behaviour, 2008; 90: 501-511.

Teeling JL, Perry VH. Systemic infection and inflammation in acute CNS injury and chronic neurodegeneration: Underlying mechanisms. Neuroscience, 2008; 158: 1062-1073.

Terramani TT, Eton D, Dui PA, Wang Y, Weaver FA, Yui H. Human macrovascular endothelial cells: Optimization of culture conditions. *In Vitro* Cell Developmental Biology-Animal, 2000; 36: 125-132.

The recruitment, adherence and diapedesis of neutrophils during an inflammatory response. www.biogeonerd.blogspot.com/2012/04/leukocytes-and-antibodies.html.com.

The umbilical cord. http://findlaw.doereport.com/generateexhibit.php.

Tizard ER. Immunology, an introduction, Third E. Saunders College Publishing, 1992.

Tong-Rong J, Shu-Ting S, Hsin-Ying W, Mei-Hsiu L. Suppressive effects of cannabidiol on antigen-specific antibody production and functional activity of splenocytes in ovalbumin-sensitized BALB/c mice. International Immunopharmacology, 2007; 7: 773-780.

Turgeon ML. Immunology and serology in laboratory medicine, Second Edition. Mosby, 1996.

Valk PJ, Vankan Y, Joosten M, Jenkins NA, Copeland NG, Lowenberg B, Delwel R. Retroviral insertions in Evi12, a novel common virus integration site upstream of Tra1/Grp94, frequently coincide with insertions in the gene encoding the peripheral cannabinoid receptor Cnr2. Journal of Virology, 1999; 73: 3595-3602.

Van Rensburg CEJ, Anderson R, Jooné G, Myer MS, O'Sullivan JF. Novel teramethylpiperidine-substituted phenazines are potent inhibitors of P-glycoprotein activity in a multidrug resistant cancer cell line. Anti-Cancer Drugs, 1997; 8: 708-713.

Wheller SK, Perretti M. Dexamethasone inhibits cytokine-induced intercellular adhesion molecule-1 up-regulation on endothelial cell lines. European Journal of Pharmacology, 1997; 331: 65-71.

Wiley JL, Razdan RK, Martin BR. Evaluation of the role of the arachidonic acid cascade in anandamide's *in vivo* effects in mice. Life Sciences, 2006; 80: 24-35.

Yi-Yuan Y, Chaur-Jong H, Su-Mei C, Tzu-Yi T, Sy-Jye L. Aspiren inhibits monocytes chemoattractant protein-1 and interleukins-8 expression in TNF-α stimulated human umbilical vein endothelial cells. Atherosclerosis, 2004; 174: 207-213.

Young B, Heath JW. Wheater's Functional Histology, a text and colour atlas, Fourth Edition. Elsevier, 2000.

Zurier RB, Rosetti RG, Burstein SH, Bidinger B. Suppression of human monocyte interleukin-1ß production by ajulemic acid, a nonpshycoactive cannabinoid. Biochemical Pharmacology, 2003; 65: 649-655.

Annexure A: Patient information leaflet and informed consent for the use of their umbilical cord

Project title

An in vitro investigation of the effects of Rimonabant (a cannabinoid CB_1 receptor antagonist) on cell adhesion and inflammatory associated cytokine production.

Introduction

You are invited to volunteer for a research study. This information leaflet is to help you to decide whether you would like to participate. Before you agree to take part in this study you should fully understand what is involved. If you have any questions, which are not fully explained in this leaflet, do not hesitate to ask the investigator. You should not agree to take part unless you are completely happy about all the procedures involved.

The purpose of this study

The purpose of this study is to test whether Rimonabant could be used to relieve the symptoms associated with inflammatory diseases such as autoimmune diseases and allergies. Rimonabant will be tested on cells obtained from human umbilical vein cells. The umbilical cord is traditionally disposed of in biowaste containers. You are however asked to donate your baby's umbilical cord in order to do research on umbilical cord cells. This is not genetic research and neither you nor your baby will benefit from the research. These cells are used because they express the correct markers for inflammation for the *in vitro* (test tube) experiments.

Risk and discomfort involved

There will be no risk or discomfort involved when donating your baby's umbilical cord.

Has this study received ethical approval?

The study protocol was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria and written approval has been granted by that committee.

Confidentiality

All information obtained during the course of this study is strictly confidential. Data that may be reported in scientific journals will not include any information, which identifies you as a donor in this study. Any information uncovered regarding your state of health as a result of your participation in this project will be held in strict confidence.

Informed consent

I hereby confirm that I have been informed by the investigator, Ms _____about the nature, conduct, benefits and risks of this study. I have also received, read and understood the above written information (Patient Information Leaflet and Informed Consent) regarding the project.

I am aware that the results of the study, including personal details regarding my sex, age, date of birth, initials and surname will be anonymously processed into a study report.

I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.

Patient's name _____
(Please print)

Date

Patient's signature _____ _____

Investigator's name _____
(Please print)

Date

Investigator's signature _____ _____

I, Dr_____ herewith confirm that the above mentioned patient has been informed fully about the nature, conduct and risks of the above project.

Witness's name _____

Witness's signature_____ Date_____

Verbal patient informed consent

I, the undersigned, Dr _____have read and have explained fully to the patient, named _____and/or his/her relative, the patient information leaflet, which has indicated the nature and purpose of the project in which I have asked the patient to participate. The explanation I have given has mentioned both the possible risks and benefits of the project.

I hereby certify that the patient has agreed to participate in this study.

Patient's name _____
(Please print)

Investigator's name _____
(Please print)

Date

Investigator's signature _____ _____

Annexure B: Ethics Committee Approval

University of Pretoria

Faculty of Health Sciences Research Ethics Committee
University of Pretoria
Tel: (012) 339 8619 Fax: 086 5516847
E Mail deepeka.behari@up.ac.za
Date: 28/06/2006

Soutpansberg Road
MRC-Building
Room 2 - 20

Private Bag x 385
Pretoria
0001

Number : **S39/2006**

Title : An investigation of the possible anti-inflammatory properties of Acomplia (Rimonabant, a cannabinoid CB1 receptor antagonist)

Investigator : A Bouwer, Department of Pharmacology, University of Pretoria (SUPERVISOR: PROF J R SNYMAN)

Sponsor : **Enerkom Research Fund**

Study Degree : **MSc (Pharmacology)**

This Student Protocol has been considered by the Faculty of Health Sciences Research Ethics Committee, University of Pretoria on 27/06/2006 and provisional approval herewith given, pending approval from CEC of Pretoria Academic Hospital and the head of the Department of Obstetrics and Gynaecology.

Mr K P Behari	B.Proc. (KZN); LLM – (UNISA)
Prof J A Ker	Deputy Dean
Advocate AG Nienaber	(female)BA(Hons) (Wits); LLB; LLM (UP); Dipl.Datametrics (UNISA)
Prof V O.L. Kanssell	MBChB: MFGP (SA); M.Med (Chir); FCS (SA): Surgeon
Dr M E Kenoshi	MBChB: DTM & H (Wits): C.E.O. of the Pretoria Academic Hospital
Prof M Kruger	(female) MBChB.(Pret): Mmed.Paed.(Pret); PhDd. (Leiven)
Dr N K Likibi	MBChB.; Med.Adviser (Gauteng Dept.of Health)
Dr F M Mulaudzi	(female) Department of Nursing
Mrs E.L. Nombe	(female) B.A. CUR Honours; MSC Nursing - UNISA
Snr Sr J. Photoli	(female) BCur (E.L.A) Senior Nursing-Sister
Dr L Schoeman	(female) Bpharm. BA Hons (Psy), PhD
Prof H.W. Pretorius	MBChB; M.Med (Psych) MD: Psychiatris:
Prof J.R. Snyman	MBChB. M.Pharm.Med: MD: Pharmacologist
Dr R Sommers	(female) MBChB; M.Med (Int); MPhar.Med;
Prof TJP Swart	BChD, MSc (Odont), MChD (Oral Path) Senior Specialist; Oral Pathology
Prof C W van Staden	MBChB; Mmed (Psych): MD; FTCL; UPLM; Dept of Psychiatry

Student Ethics Sub-Committee

Mrs E Ahrens	(female) B.Cur
Dr L Schoeman	(female) Bpharm. BA Hons (Psy), PhD
Dr R Sommers	SECRETARIAT (female) MBChB: M.Med (Int); MPharMed
Mrs N Lizamore	(female) BSc(Stell), BSc (Hons) (Pret),MSc (Prec); DHETP (Prec)
Prof R S K Apatu	MBChB(Legon); PhD(Cambridge)
Dr S I Cronje	DD (UP) – Old Testament Theology
Dr M M Geyser	(female) BSc; MBChB; BSc HONS (Pharm); Dip PEC: MpraxMed
Mrs D Millard	(female) Biur LLB LLM LLD (UJ)
Dr A.M Bergh	(female) BA (*cum laude*), Rand Afrikaans University BA (Hons) (Linguistics), University of Stellenbosch Secondary Education Diploma (*cum laude*), University of Stellenbosch BA (Hons) (German) (*cum laude*), University of South Africa (Unisa) BEd (Curriculum Research and Non-formal Education) (*cum laude*), University of Pretoria PhD (Curriculum Studies), University of Pretoria

pp PROF J R SNYMAN
MBChB, M.Pharm.Med: MD: Pharmacologist
CHAIRPERSON of the Faculty of Health Sciences Research Ethics Committee - University of Pretoria

DR L SCHOEMAN
Bpharm, BA Hons (Psy), PhD
CHAIRPERSON of the Faculty of Health Sciences Research Students Ethics Committee – University of Pretoria

Annexure C:

Patient information leaflet and informed consent for the use of healthy donor blood

Project title

An in vitro investigation of the effects of Rimonabant (a cannabinoid CB_1 receptor antagonist) on cell adhesion and inflammatory associated cytokine production.

Introduction

You are invited to volunteer for a research study. This information leaflet is to help you to decide whether you would like to participate. Before you agree to take part in this study you should fully understand what is involved. If you have any questions, which are not fully explained in this leaflet, do not hesitate o ask the investigator. You should not agree to take part unless you are completely happy about all the procedures involved.

Purpose of this project

The purpose of this study is to test whether Rimonabant could be used to relieve the symptoms of inflammatory diseases such as autoimmune diseases and allergies. Rimonabant will be tested on cells obtained from human blood. You are asked to donate 120 ml of blood on which the above *in vitro* (test tube) tests can be done. The aim is not to treat or diagnose a disease but purely to do tests on the cells in the blood obtained from you. You will not benefit from this study at all.

Risk and discomfort involved

The only possible risk and discomfort involved is the taking of blood from the vein.

Has this study received ethical approval?

The study protocol was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria and written approval has been granted by that committee.

Confidentiality

All information obtained during the course of this study is strictly confidential. Data that may be reported in scientific journals will not include any information, which identifies you as a donor in this study. Any information uncovered regarding your state of health as a result of your participation in this project will be held in strict confidence.

Informed consent

I hereby confirm that I have been informed by the investigator, Ms _____about the nature, conduct, benefits and risks of this study. I have also received, read and understood the above written information (Patient Information Leaflet and Informed Consent) regarding the project.

I am aware that the results of the study, including personal details regarding my sex, age, date of birth, initials and surname will be anonymously processed into a study report.

I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study

Patient's name _____
(Please print)

Date

Patient's signature _____ _____

Investigator's name _____
(Please print)

Date

Investigator's signature _____ _____

I, Dr_____ herewith confirm that the above mentioned patient has been informed fully about the nature, conduct and risks of the above project.

Witness's name _____

Witness's signature_____ Date_____

Verbal patient informed consent

I, the undersigned, Dr _____have read and have explained fully to the patient, named _____and/or his/her relative, the patient information leaflet, which has indicated the nature and purpose of the project in which I have asked the patient to participate. The explanation I have given has mentioned both the possible risks and benefits of the project.

I hereby certify that the patient has agreed to participate in this study.

Patient's name _____
(Please print)

Investigator's name _____
(Please print)

Date

Investigator's signature _____ _____