In vitro Tests for the Diagnosis of Aspirin and Salicylate Sensitivity

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University of Wales

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DECLARATION

This work has not previously been accepted in substance for any degree and not being concurrently submitted in candidature for any degree.

Signed .......................................................... (candidate)
Date .............................................................. 5/03/2004

STATEMENT 1

This thesis is the result of my own investigation, except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

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Date .............................................................. 5/03/2004

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Date .............................................................. 5/03/2004
Dedication

To my sister Maha, who is the source of my strength and inspiration, who believed in me when others did not, and to my beloved once Aisha Khadjgha and Ftooh whose prayers and blessing always return me to the right track.
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Finally, I am deeply indebted to Kuwait Government who offered the financial support throughout the years of my study.
Abbreviations

AA    amino acid
AIA   aspirin induced asthma
ASA   acetylsalicylic acid
CAD   coronary artery disease
CD    cluster of differentiation (also designation)
CD63  platelets activation antigen.
cDNA  complementary deoxyribonucleic acid
COX   cyclooxigenase
DNA   deoxyribonucleic acid
dNTP  deoxynucleotid triphosphate
ds    double stranded
ECP   eosinophil cationic Protein
EDTA  ethylenediamino tetra-acetic acid
EtBr  ethidium bromide
FACS  fluorescence activated cell sorting
FEV₁  forced expiratory volume in first second
FITC  fluorescein isothiocyanate
IgE   immunoglobulin E
IL-4  interleukin-4
L-ASA lysine-acetylsalicylic acid
MBP   major basic protein
mg    milligram
μg    microgram
ml    milliliter
μl    microlitre
min   minute(s)
MM6   Monocytic mac cell line
MOPS  3-(N-morpholino) proane-sulphonic acid, sodium salt
mRNA  messenger ribonucleic acid
NAR   nasal airway resistance
NART  nasal airway resistance test
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>NSAIDs</td>
<td>non steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>P</td>
<td>significant probability</td>
</tr>
<tr>
<td>Pa</td>
<td>pascal</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerytherin</td>
</tr>
<tr>
<td>PEFR</td>
<td>peak expiratory flow rate</td>
</tr>
<tr>
<td>Per-CP</td>
<td>peridinin chlorophyll protein</td>
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<tr>
<td>pg</td>
<td>picogram</td>
</tr>
<tr>
<td>PGF$_{2\alpha}$</td>
<td>prostaglandin F 2 alpha.</td>
</tr>
<tr>
<td>PHA</td>
<td>phytohemagglutinin</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>rpm</td>
<td>revolution per minute</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>ss</td>
<td>single stranded</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate-EDTA</td>
</tr>
<tr>
<td>V</td>
<td>volts</td>
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Sensitivity to aspirin, non-steroidal anti-inflammatory drugs (NSAIDs) and also dietary salicylate is an increasingly important and observed phenomenon. The reliable diagnosis of individuals with these disorders is of great importance, given the use of these drugs as a prophylactic medication. Many of the symptoms seen in patients associated with this sensitivity to these drugs include dermatitis, nasal polyps, persistent cough, rhinitis, skin problems, stomach irritation, swelling of face and urticaria are also seen in salicylate intolerance. These symptoms are similar to those seen in the allergic patient, however, the reaction to salicylate is not an allergy. A major clinical problem is that no current method of food intolerance or allergy in vitro testing and diagnosis has currently been established to predict those people with this condition.

This study was designed to search for new markers, which could be used to predict with high specificity and sensitivity, subjects with aspirin induced asthma (AIA). This was done by investigating patients with known aspirin sensitivity and comparing them with healthy individuals and atopic non-aspirin sensitive patients. All subjects used (male/females- age range 21-68 years old) were divided into 4 groups (atopic aspirin sensitive, non-atopic aspirin sensitive, atopic non-aspirin sensitive, and healthy volunteers) according to the clinical history and confirmed positive skin test to common allergens. Flow cytometer was used to assess the role of activated basophils in AIA and detect the expression of CD63 in their blood after stimulation of the cells with L-ASA. A posterior rhinomanometer was used to assess a challenge technique that was sensitive to local stimulation by L-ASA. Cytokine (IL-4) expression in lymphocytes was determined using ELISA and IL-4 messenger RNA levels investigated using RT-PCR. The release of PGF$_{2\alpha}$ from plasma protein on those patients was detected by ELISA. The results obtained in this study, suggest that up regulation of CD63 expression by aspirin and Nasal airways resistance test (NART) following aspirin challenge, may prove to be useful diagnosis tool in these patients. The study also indicates that lymphocyte responses as evidenced by IL-4 release are not affected by salicylate sensitivity. The significance of these results is also discussed.
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Chapter One

General introduction
1. Overview

Sensitivity to aspirin (acetylsalicylic acid) (ASA) is a distinct clinical syndrome, which is characterized by an adverse reaction to aspirin. This condition is becoming increasingly recognized as the use of aspirin as a prophylactic reaction becomes more widespread. Many patients suffer a myocardial infarction (MI) each year; those who survive are significantly at risk of dying (33%) in the month following the myocardial infarction, and another 10% in the first year post-infection (Cambria-Kiely and Gandhi, 2002). There is good evidence that the long-term use of aspirin reduces mortality in high-risk patients, as it prevents platelet aggregation, thus inhibiting both primary and secondary causes of MI. A single 75mg-100mg dose of ASA can completely block thromboxane (TXA₂) production for the life of a platelet. Because platelets lack the machinery necessary to synthesis new protein, the defect induced by aspirin cannot be repaired during their life span (approximately 8 -10 days) (Szczeńlicki et al, 2001, Douglas et al, 1993).

Low doses of ASA protect against thrombotic occlusion of coronary arteries, cerebral arteries and vascular grafts. However, many patients with coronary artery disease (CAD) are denied treatment with aspirin because of a history of sensitivity to aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs) causing them to develop asthma, urticaria and angioedema. These patients can be acutely sensitive to small doses of aspirin. Aspirin triggers asthma in these individuals by inhibiting the cyclooxygenase pathway of arachidonic acid metabolism (Patrono1994, Szczeńlicki et al, 2001). Aspirin causes a special type of asthma, termed aspirin-induced asthma (AIA). Nearly one third of all patients with documented myocardial infarctions are not prescribed aspirin when they are discharged from hospital because they have a
history of aspirin sensitivity and this can be therapeutically disadvantageous. However, 15% of those patients are unaware that they are sensitive to aspirin (Szczeklik et al, 2001). This is because there is no in vitro test to identify them, and the mild reactions to aspirin are generally not recognized due to the delay between ingesting the aspirin and the onset of symptoms (Schaefer and Gore, 1999).

AIA is an aggressive inflammatory disease of the mucosa that causes increased severity of asthma, and rhinitis attacks. These studies suggest that about 10-23% of patients with nasal polyps are sensitive to these drugs. Patients experience a range of symptoms from urticaria, angiodema or anaphylaxis after ingesting aspirin or non-steroidal anti-inflammatory drugs (NSAIDs) (Szczeklik et al, 2000, Feldman et al, 2000). Beside these, there are an unidentified percentage of individuals who respond acutely to these drugs although they have no personal or family history of allergy, no response to skin prick testing to all aeroallergens and normal serum levels of total and specific IgE. These patients are termed (non-atopic)(Cotran et al, 1999). It is important to define this group since 10-40% of asthmatics are non-atopic (Pearson and Suarez-mendez, 1990, Schaefer and Gore, 1999, Mita 2001).

Since there is no reliable in vitro test to identify aspirin sensitive individuals, one has to rely on oral challenge tests. Oral challenge with aspirin and NSAIDs, caused bronchoconstriction in 19% of adult asthmatic patients and 10-20% of both children and adults (Cowburn and Sladek, 1998). However, oral challenge testing is time consuming, unpleasant for patients with asthma, and too dangerous for patients who develop angioedema (Pierzchalska et al, 2000). Furthermore, aspirin induced adverse skin reactions cannot reliably be diagnosed based on history alone, since only 21% of patients with a clear cut history of provoked skin eruptions had positive oral
provocation tests (Wedi and Kapp, 2000). These two techniques (aspirin challenge and skin test) cannot provide reliable predictive tests for drug allergies. Therefore, it would be beneficial to find new objective reproducible markers for aspirin sensitivity that can be used to develop an effective test for identifying those patients that are sensitive to aspirin (Vervloet and Durham, 1998, Wedi and Kapp, 2000). Such markers could be used not only to identify this group, but also to obtain information about the mechanism underlying this syndrome, and the host response (Wedi and Kapp, 2000).

Unwanted immune responses to aspirin and other NSAIDs are similar to allergic or type I hypersensitivity reactions. The exact mechanisms involved are still unclear, and since drug-specific IgE assays are unlikely to be useful, there is no currently reliable, sensitive and specific biochemical diagnostic test that can identify the population at risk. (Abuaf et al, 1999, Cozon et al, 1999, May et al, 1999).

1.1 Risk factors implicated in AIA.

In general, risk factors associated with the disease include genetic predisposition, family history, dietary or environmental exposures that can cause a person to develop symptoms. Although these factors can increase a person’s risk, they do not necessarily cause the disease, and in fact a combant of these factors may be necessary to (Vervloet and Durham, 1998, Puijenbroek et al, 2002)

1.1.1 Virus infection

Initial reports indicate a higher incidence of viral infection is associated with aspirin induced asthma (AIA). The first symptoms of this condition are often a flu-like viral respiratory infection, followed by a prolonged and perennial rhinitis (Lee 1993, Plaza 1995). However, there is no clear explanation offered as to how a virus infection
could be linked with the cyclo-oxygenase mechanisms. One hypothesis suggests that aspirin induced asthma results after long-term exposure to a virus, where cytotoxic lymphocytes are produced. PGE₂ (produced primarily by pulmonary alveolar macrophages) will suppress the activity of these cells. Cyclo-oxygenase analgesics will block PGE₂ production, and allow cytotoxic lymphocytes to attack and kill target (virus infected) cells. During this reaction lysosomal enzyme, toxic oxygen intermediates and mediators will be released, which will precipitate asthma. Avoiding anti-cyclo-oxygenase activity may therefore prevent acute attacks. However, this will not stop the chronic viral infection. A study showed those with Rey's syndrome should avoid aspirin, this is a rare disorder in children, it is a combination of a liver disorder and encephalopathy, which can follow an acute viral illness and has a 20-40% mortality rate. However, there is no clear implication for the role of aspirin in the causation of this syndrome (Brody 1998, Rang 1999, Szczeklik and Sanak, 2000).

1.1.2 Heredity

Adverse drug reactions occur mainly in middle age and are twice as common in women as in men, it is thought that genetic factors play an important role in these conditions (Vervoet and Durham, 1998). However, Schaefer and Gore found that, while a few of the families they studied had “aspirin trait” which is asthma and nasal polyposis. Most of them had no family history of asthma or aspirin sensitivity (Schaefer and Gore, 1999). Szczeklik, 1986 found only two cases of familial intolerance to aspirin among 600 patients with proven aspirin induced asthma. Szczeklik suggests that HLA type, that predisposes people to reactions to aspirin, is HLA-DQw2. About two-thirds of the patients with ASA had an increased frequency of HLA-DQw2, while those with other types of asthma and a good tolerance to aspirin did not (Sanak and Szczeklik, 2000, Vervoet and Durham, 1998). Several
other studies have confirmed this finding, however, in contrast some studies have shown no significant differences in the frequencies of any DRB1, DQA1, or DQB1 alleles in aspirin sensitive patients and asthma and aspirin-tolerant control (Nizankowska et al, 1997, Mullarkey et al, 1986). Some studies attempt to study other allele such as (HLA-DRB1), the data suggested that this allele was associated with aspirin-induced asthma, exclusively in atopic patients (Quiralte et al, 1999, Vervloet and Durham, 1998). Several studies have found several times more infiltrating eosinophils which are a site of cysteinyl leukotriene synthesis in bronchial biopsies taken from patients with aspirin induced asthma (AIA), than in aspirin tolerant patients (Kawagishi 2002, Szczeklik and Nizankowska, 2000). Other investigation have shown an over expression of LTC₄ synthesis, perhaps because of genetic polymorphism of the gene encoding LTC₄, in support of this they also found that the -44C allele (lacking cytosine 444) is more common in aspirin sensitive asthmatics than in patients with aspirin tolerant asthma (ATA) and normal subjects (Szczeklik and Sanak, 2000, Szczeklik and Nizankowska 2000). Although genetic factors may play a role and alter the risk of developing an allergic diseases, it is the change in the enviroment that most likely to account (Holgate 2001).

1.1.3 Gender

Several reports indicate that AIA is more common in women (Pearson and Suarez-mendez, 1990, Szczeklik et al, 2000). Studies have indicated that disease development seems to be constant throughout the life in males, in contrast to females where it is increased 6-fold during the re-productive years. This may be attributed to a hormonal effect, which one study related to gonadal hormone control (Maggi et al, 1981).
Several reports concern the effect of gender on the disposition and pharmacokinetics of aspirin and salicylate. These reports found that the time taken to reach maximum plasma concentration of salicylate was longer in females. However, the administration of sodium salicylate to male and female subjects showed no gender dependent differences in the time taken to reach the peak concentrations or respective volumes of distribution for aspirin and other NSAIDs (Pearson and Suarez-mendez, 1990, Schiavino et al, 2000, Zambraski and Dunn, 1992).

1.2 Allergy

Allergy is an abnormal reaction to ordinarily harmless substances, regardless of whether that reaction was exaggerated as in hypersensitivity, or diminished as in immunity. The word “allergy” derived from the Greek words allos meaning other and ergon meaning work or action. It has been suggested that “allergy” be replaced by the more specific designation “disease of immediate hypersensitivity”.

IgE is immunoglobulin class mediates the immediate hypersensitivity reaction that is involved in allergic reactions. IgE functions by sensitising the effector cell, which, in response to stimulus, secretes mediators of immediate hypersensitivity. Two common effector cells are mast cells and basophils. Both of these all types have thousands of receptors for IgE molecules. Re-exposure to the same antigen results in binding of the antigen to cell surface, IgE, triggering the release of intracellular granules that spill out their contents onto neighbouring cells, including blood vessels and nerve cells (Bochner and Sterbinsky, 1991). Mast cells and basophils may also be triggered by several other stimuli, such as complement components C5a and C3a (anaphylatoxins), both of which act by binding to their receptors on the mast cell membrane, some
drugs such as codeine and morphine, mellitin (present in bee venom), and physical stimuli such as cold, heat and sunlight (Abrahamsen et al, 2001, Kumar et al, 1992).

1.2.1 Type I: immediate (anaphylactic hypersensitivity)

Type I hypersensitivity is the rapidly occurring reaction that follows the combination of an antigen with an antibody. This happens when the antigenic material which is not in itself noxious (such as grass pollen, products of dust mite, food or some drugs) evokes the production of IgE, which binds to the surface of mast cells and combines and cross-links with it. This will leads to the release of pre-formed mediators including histamine, heparin, platelet activation and the biochemical cascade producing membrane active “fusogens” such as lyso-phosphatidic acid, which will facilitate granular membrane fusion and degranulation of the mast cells (Oettgen and Geha, 1999, Moodycliffe et al, 1995, Sainte-Laudy et al, 1998).

The cyclo-oxygenase and 5-lipoxygenase pathways form arachidonic acid metabolites during the mast cell and basophil activation. This results in the release of newly synthesized mediators such as prostaglandins, leukotrienes and thromboxanes that produce an acute inflammatory reaction. Recently, type I immediate hypersensitivity has been defined as having two phases; the first is an initial response, characterized by vasodilatation, vascular leakage, and smooth muscle spasm which usually becomes obvious within 5-30 minutes after exposure to an allergen and tends to drop in 60 minutes, and the second or “late phase” reaction occurs 2-8 hours later without additional exposure to antigen and can last for several days (Puijenbroek et al, 2002, Rang et al, 1996). This late phase is characterized by the intense infiltration of eosinophils, neutrophils, basophils and monocytes into tissues as well as tissue destruction in the form of mucosal epithelial cell damage. Type I responses may be
localised to the nose (hay fever), skin (urticaria), the bronchial tree (the initial phase of asthma) and the gastrointestinal tract (Figure 1.1)(Moodycliffe et al, 1995, Cotran et al, 1999).

**Figure 1.1: Type I immediate hypersensitivity**

The activation of mast cell after cross binding with IgE and the antigen leads to the granules mediators’ release. (Taken from Roitt 1991)

**1.2.2 Cells involved in allergic reactions**

**1.2.2.1 Mononuclear cells**

These are composed of two types of cells. The first type are the monocytes and macrophages these process antigens for presentation to lymphocytes, as well as stimulating mediator release from mast cells. The second type are the lymphocytes,
the primitive cells that originate from bone marrow develop into two major lymphocyte subsets.

B cells differentiate in the bone marrow and enter to the circulation as mature cells. T cells require a period of differentiation in the thymus. B-lymphocytes produce antibody molecules under the influence of T cells. T cells regulate the switching of B cells through two signal process immunoglobulins (Frew 1998). As T cells develop, they become either CD4+ helper or CD8+ cytotoxic. It is the CD4+ T -cells that are involved in the regulation of immune responses. CD4+ helper T cells have been subdivided into two groups, Th1 and Th2. The function that these helper T- cells (Th1 - Th2) provide to B cells activates molecules called cytokines, which bind to and stimulate, B cells and other lymphocytes and monocytes (Minty et al, 1997)

Several studies have found that CD4+ T cells are the only cells in the immune system that can both directly recognize allergic peptides and can release interleukins. Furthermore, CD4+ T cells direct humoral immunity and immediate hypersensitivity reactions, which provides defences against extra cellular pathogens. Th1 and Th2 cells represent the later stage of differentiation from Th0 cells. When Th2 activated a number of intermediate cytokine profile are displayed such as IL-4, IL-10 which activate the B cell to switch to IgE, also these cytokines (IL-4, IL-10) are important to mast cells proliferation and granules degradation (Seah et al, 2001). IL-5 is another cytokine that produced by Th2, and has an important role in eosinophil proliferation (Table 1.1)(Romagnani 2001, Broide 2001).
Table 1.1: The properties of $T_H^1$ and $T_H^2$ cells

<table>
<thead>
<tr>
<th>Type of cell</th>
<th>$T_H^1$</th>
<th>$T_H^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines produced</td>
<td>IL-2, IFN-$\gamma$</td>
<td>IL-4, IL-5, IL-6, IL-10</td>
</tr>
<tr>
<td>Cell types helped</td>
<td>Macrophages, Cytotoxic T cells</td>
<td>B cells</td>
</tr>
<tr>
<td>Immunity generated</td>
<td>Cell- mediated</td>
<td>Antibody – mediated</td>
</tr>
<tr>
<td>Cells suppressed</td>
<td>$T_H2$</td>
<td>$T_H1$</td>
</tr>
</tbody>
</table>

Through the inflammatory cascade mechanism, IL-4 regulates differentiation of naïve T (H) cells to develop a $T_H^2$ phenotype and stimulates B cells to produce IgE. The roles of lymphocytes and the production of cytokines by them in the human body will be discussed in more details in chapter (4-5).

1.2.2.2 Basophils

Basophils are small polymorphonucleated cells characterized by their intracellular basophilic granules. Several studies have suggested a major role for basophil as producers of various biologically active mediators. It is well known that basophils possess a marked modulating activity of inflammatory reactions, immune responses and cellular proliferation. They differ from other cells such as mast cells and eosinophils in three ways. Firstly, in their response to diverse agonists, secondly, in
the mediators they produce and thirdly, by their expression of large amounts of high affinity IgE receptor FcεRI (Bühring et al, 1999, Toba et al, 1999).

Basophils are an important source of inflammatory cytokines. Although T cells are the main source of cytokines such as IL-4 and IL-13, several studies have shown that the quantity of cytokines produced by basophils is much greater than that of T cells when they have been stimulated (Chan et al, 1996, Shimizu et al, 1998). Basophils can be identified using several other markers e.g. CD33, CD17, CD61, CD45 and CD63 that are expressed by human basophils (Knol et al, 1991, Monneret et al, 1999)

CD63 is a lysosomal membrane glycoprotein expressed by platelets, monocytes, and macrophages as well as being weakly expressed by T cells, B cells and basophils. The biological function for CD63 is not yet known; but its broad distribution suggests it may have a role in signalling between platelets and other cells. CD63 is weakly expressed by resting basophil in non-atopic subjects. However, activated basophils express large amounts of CD63 (Moneret-Vautrin 1999, Taylor 1996, Toba 1999). Several studies have used CD63 as a marker with the facilitated use of flow cytometry as a more sensitive technique for measuring cell activation after stimulation with specific antigens (Talyor 1996, Wedi et al, 2000).

For the purpose of this study CD63 analysis with special attention to flow cytometry will be discussed in more detail in chapter two.

1.2.2.3 Eosinophils

Eosinophils are granulocytes whose cytoplasm contains eosinophilic granules. Their production in the bone marrow is modulated by a number of cytokines including IL-3, IL-5, IL-6, and GM-CSF (Granulocyte-macrophage colony-stimulating factor). Eosinophils are currently regarded as the effector cells responsible for much of the
pathology of asthma. It has long been known that atopy in general, and asthma in particular is associated with blood and lung eosinophilia (Giembycz et al, 1999).

Eosinophils are important in diseases where no role for IgE has been detected, such as "non-allergic" asthma. These include asthma that is due to sensitisation to aspirin and other small molecules, as well as intrinsic asthma (Greiff et al, 2001, Paggiaro et al, 1990).

Several studies have shown that, eosinophils are increased in bronchoalveolar lavage fluid from asthmatics compared to non-asthmatics (Djukanovic et al, 1990, Bousquet et al, 1990). Eosinophils do not express the FceRI receptor, thus they must be recruited into both allergic and non-allergic inflammation by mechanisms other than IgE. This is probably mainly by means of cytokines (especially IL-5) produced by T-cells and mast cells (Bandeira-Melo, 2001). They secrete a number of proteins; the most important of these is major basic protein (MBP) that exhibits potent cytotoxic activity (Gleich 1990). Cationic protein is another eosinophil protein, which exhibits ribonuclease activity. A third is eosinophil peroxidase, this is cytotoxic in itself, but more so in the presence of hydrogen peroxide and halides. It can also cause mast cell degranulation (Greenfeder et al, 2001)

1.2.2.4 Mast cells

Mast cells are the main effector cells involved in type I hypersensitivity responses. They are bone marrow derived cells that are widely distributed in connective tissues, predominantly near blood vessels and nerves and in subepithelial sites, and occurring in lymph nodes. They require T cells for their growth and differentiation (Nomura et al, 2001, Buhring et al, 1999). Mast cell cytoplasm contains membrane bound granules that contain a variety of biologically active mediators. These cause
vasodilatation, vascular permeability and smooth muscle contraction as well as leukocyte chemotaxis that leads to clinical manifestations, such as rhinitis, asthma, and in severe cases, anaphylaxis (Kumar et al, 1992, Reeves et al, 1996).

Activated mast cells secrete molecules that can attract inflammatory cells (such as eosinophils) and enhance the allergic reaction. One such mechanism is the activation of biochemical pathways that metabolise a component of cell membranes called arachidonic acid. Arachidonic acid can be metabolised via two major pathways and by two different enzyme systems, cyclooxygenase and lipoxygenase, the end products of which are prostaglandins and leukotrienes respectively. These are capable of enhancing inflammation that is characteristic of this part of the allergic reaction. Recently, a new test measuring serum mast cell tryptase has become available. Serum tryptase levels rise rapidly during anaphylaxis. This test has been found to be useful, particularly when an anaphylactoid reaction occurs during anaesthesia, as a reliable index of mast cell activation (Bosso et al, 1991, Varga et al, 1999).

1.2.3 Mediators

1.2.3.1 Histamine

There are two types of primary mediators: (1) those that are rapidly released and act early in the hypersensitivity reaction such as histamine; and (2) those that make up the granule matrix and are released slowly (these include heparin, neutral proteases such as tryptase)(Bosso et al, 1991). These factors are believed to participate in the late phase reaction of type I hypersensitivity.

Histamine released from basophils, mast cells in skin, the nasal cavity, and bronchial gastro intestinal mucosa (Bochner 2000). The interaction of histamine with receptors on blood vessels causes neighbouring cells to leak, leading to fluid collection,
swelling and increased flushing of skin and stuffiness in the nose. Histamine causes smooth muscle contraction in the bronchi, which may make breathing difficult during an asthma attack; it also stimulates the pain receptors, causing the itchiness in nose, eyes, and throat (Asad et al, 1987, Crockard and Ennis, 2001b, Sanz et al, 2001).

1.2.3.2 Leukotrienes

Leukotrienes are important in the pathogenesis of type I hypersensitivity. Leukotriene C₄ and leukotriene D₄ are the most potent vasoactive and spasmogenic agents known. One study observed that leukotrienes are 100 to 1,000 times more potent than histamine in causing constriction of bronchial smooth muscle. The ingestion of aspirin or NSAIDs increases the excretion of urinary LTE₄, which is responsible for inflammation by stimulating the local leakage of fluid from blood vessels (Moneret-Vautrin et al, 1999, Lam and Austen, 2000, Simmet et al, 1985 Sanak et al, 2000).

1.2.3.3 Prostaglandins

Prostaglandins initiate the secretion of thick sticky mucus in the bronchial tree, and contribute to itchiness and pain associated with the allergic reaction. PGD₂ and PGF₂α are the most abundant prostaglandins produced. They cause intense bronchospasm as well as increased mucus secretion. Prostanoids are released from different organs and tissue with specific patterns of enzymes determining the amount of the end products formed in the synthetic chain from arachidonic acid via the endoperoxides PGG₂-PGH₂. They are produced as a result of any damage or injury and their release sometimes serves as an alarm signal by its stimulation of pain receptors actions (Kowalski et al, 2000).
Prostanoids play a central role in inflammation as well as regulate other critical physiological responses. Prostaglandins are involved in human blood clotting, ovulation, initiation of labour, bone metabolism, nerve growth and development, wound healing, kidney function and many more (Williams et al, 1991).

The effects of NSAIDs and other related drugs such as aspirin and indomethacin are not restricted to the inhibition prostaglandin release. NSAIDs have other actions such as the inhibition of serotonin release and platelet release, and also a decrease in vascular permeability. The suppression of prostaglandin synthesis in normal human physiology through the inhibition of COX can lead to unwanted side effects, and many adverse reactions. For example, 25% of individuals taking NSAIDs for short periods of time experienced gastrointestinal and renal side effects (Szczechlik et al, 1998, Bennett 2000, Williams1991).

In chapter three, the role of prostanoids in human body will be covered in more detail.

1.2.3.4 Platelet-activating factor (PAF)

PAF causes platelet aggregation, which leads to calcium-dependent release of histamine the degranulation of neutrophils and the contraction of smooth muscle. Although its production is initiated by the activation of phospholipase A₂, it is not a product of arachidonic acid metabolism (Dubois et al, 1998, Ameisen et al, 1985).

1.2.3.5 Cytokines

Other identified chemical mediators of the allergic reaction are cytokines. There is increasing evidence that mast cells and basophils can produce a variety of cytokines, including TNF-α, IL-1, IL-5, IL-6, and IL-4. Cytokine secretion by T cells is a critical component of specific immunity (Schroeder et al, 1998, Devouassoux et al, 1999).
Among the many cytokines produced, IL-4 has an important role in the up regulation of IgE production and the expression of MHC class II molecules on B cells. IL-4 is known to promote the differentiation of Th0 cells into Th2 cells, and is a critical determinant of the outcome of infection or autoimmunity (Bullens 1998, Cron et al, 1999).

IL-5 has been found to have an important role in the differentiation and activation of eosinophils. Both IL-4 and IL-5 are therefore essential factors in the development of allergic disease. The detection of cytokines may prove to be useful the diagnosis of the disease states. The expected role of these cytokines in ASA will be discussed later chapters four and five.

1.3 Salicylate products

1.3.1 Chemical products (Aspirin)

Last century, when the interest in salicin (the origin of salicylate ingredient) and its derivatives increased, it was found that there were many different naturally occurring sources of Salicin in plants.

Salicin can be readily found in a number of different plants, ranging from large trees to shrubs and ornamental flowers. Salicin can be found in:

- Willow trees both the bark and the leaves.
- Oil of wintergreen
- Sweet Birch
- A variety of roses, as well as Spirea plants, where part of the name aspirin is derived from.
1.3.1.1 Discovery of Aspirin

The Greeks discovered, around the fifth century BC, that a substance in the bark of the willow tree, now known as Salicin, could relieve pain (Tramer 2000). Originally the Greeks used the bark of the white willow tree, (*Salix alba*), as their source of Salicin. Around the fourth century BC, Hippocrates used a bitter powder obtained from willow bark to ease aches and pains and reduce fever. He also advocated the chewing of willow leaves to relieve the pains of childbirth. In fact, acetylsalicylic acid, the main ingredient in all aspirin compounds found today, is a derivative of a group of compounds called the salicylates whose origins lie in the naturally occurring compound salicin (Elwood et al, 1997).

Over 100 years ago, a German industrial chemist, Felix Hoffman, discovered a stable form of acetylsalicylic acid, the active ingredient of aspirin. Hoffman was motivated by the suffering of his father, who had severe arthritis, but could not tolerate the severe stomach irritation associated with sodium salicylate, the standard drug of the time for treating chronic rheumatoid arthritis and gout, as well as an antiseptic compound. In the forms available then, the large doses of salicylate used to treat arthritis (6 to 8 grams a day) commonly irritated the stomach lining, and many patients, like Hoffman's father, simply could not tolerate them. Hoffman started looking for a less acidic formulation. His search led him to synthesize acetylsalicylic acid (ASA), a compound that appeared to share the therapeutic properties of other salicylates and might cause less stomach irritation (Vane 2000,Sneader 2000). The drug succeeded in easing his father's pain and inflammation; it proved more palatable, less irritating to the stomach, and more effective. Bayer patented the name and
commenced in marketing the product in 1899. It was a huge success and it quickly became the world's most popular pain reliever (Vane 2000, Elwood et al, 1997).

Currently, aspirin is the world’s most used drug, with more than 80 billion tablets consumed a year in the United States alone. Yet the pleiotropic effects of this drug are still to be fully realised.

1.3.1.2 Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

In the latter part of the twentieth century, non-steroidal anti-inflammatory drugs (NSAIDs), new agents with similar actions to aspirin but diverse chemical structures were discovered. Aspirin (acetylsalicylic acid) -1899 and Sodium salicylate -1875 were the first drugs of this group used clinically (Vane 2000).

NSAIDs with their diverse chemical structures share many therapeutic properties and adverse effects with Aspirin. They alleviate the swelling, redness and pain of inflammation, reduce general fever by the temperature regulation mechanisms (dilatation of superficial blood vessels, sweating), they also cure headaches and inhibit thrombocyte aggregation, but these drugs have no documented effect on the disease process itself (Rang, et al, 1999, Gøtzsche 2000). Therefore, as a result of the similarity they have tended to be regarded as a single group known as aspirin-like drugs or NSAIDs (Cashman et al, 1995, May et al, 1999).

NSAIDs such as (Ibuprofen, NaproXen and Nabumetone) are the drugs, which most commonly cause anaphylaxis. They are responsible for causing episodes in 15% of asthmatic patients (Figure1.2). NSAIDs have particularly strong O₂ – radical scavenging effects that decrease tissue damage caused by reactive oxygen radicals neutrophils and macrophages. However, the main pharmacological function for NSAIDs is the inhibition of cyclo-oxygenase (Table 1.2).
1.3.1.3 Adverse effects of these NSAIDs

All drugs are capable of producing harmful responses as well as beneficial effects. The adverse effects are either related or unrelated to the principal pharmacological actions of the drug. The unwanted effects that relate to pharmacological action are expected and well known since the chemical structure was understood in sufficient detail (Table1.1). Unrelated adverse reactions may occur when the drug is taken in excessive doses or long-term (e.g. Aspirin induced tinnitus, Aminoglycoside causes ototoxicity, and paracetamol can cause hepatotoxicity). Most drugs that produce allergic reactions are of low molecular weight and behave as haptens that are not in themselves immunogenic. However these drugs may interact and bind with proteins before being recognised by lymphocytes and form a stable conjugate that can function as an immunogen (Bircher 1999, Rang 1996, May et al, 1999).

Clinically, aspirin and other NSAIDs are among the most frequent causes of adverse drug reactions. Symptoms include skin urticaria and angioedema and resemble true allergy mediated reactions. Aspirin intolerance however, is not IgE mediated; there is no evidence that immune mechanisms that are specific for the eliciting agent are involved, and therefore the reactions seen are termed “pseudo-allergic reactions”. They are distinguished from allergic reaction, by the fact that they occur at the first administration and do not need a sensitization phase. There are five types of pseudo allergic and allergic reactions to aspirin and NSAIDs (Table1.3). The first two types of reaction are closely linked to the effects of aspirin and NSAIDs in inhibition of COX enzymes and are the result of increased arachidonic acid metabolism in 5-lipoxygenase pathways. The three other reactions are drug specific, and appear to be

Table 1.2: Classification of adverse reaction to drugs

<table>
<thead>
<tr>
<th>Reaction that may occur in normal subjects</th>
<th>Reaction that occur only in susceptible subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drugs overdose – Toxic reactions linked to excess dose or impaired excretion.</td>
<td>Drug intolerance- A low threshold to the normal pharmacological action of a drugs</td>
</tr>
<tr>
<td>Drugs side effects-Undesirable pharmacological effects at recommended doses.</td>
<td>Drug idiosyncrasy-A genetically determined, qualitatively abnormal reaction to a drug related to a metabolic or enzyme deficiency</td>
</tr>
<tr>
<td>Drugs interaction – Action of a drug on the effectiveness or toxicity of another drug.</td>
<td>Drug allergy-An immunologically mediated reaction, characterized by specificity, with recurrence on re-exposure.</td>
</tr>
</tbody>
</table>

* Pseudo-allergic reaction- A reaction with the same chemical manifestation as an allergic reaction (e.g. as a result of histamine release) but lacking immunological specificity

The unwanted effects that related to pharmacological action of the drugs.* Non-specific complement activation and non-specific histamine release may mimic type I reaction.
Table 1.3: The pseudo-allergic and allergic reaction to aspirin and NSAIDs.

<table>
<thead>
<tr>
<th>Reaction Type</th>
<th>Underlying Disease</th>
<th>Cross reactions</th>
<th>Arachidonic acid Dysfunction</th>
<th>Probable IgE</th>
<th>Possible Cellular Immunity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cross reacting respiratory</td>
<td>Rhinitis, polyps, asthma</td>
<td>ASA/NSAID</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2. Cross reacting urticaria</td>
<td>Chronic urticaria</td>
<td>ASA/NSAID</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>3. Urticaria/anaphylaxis</td>
<td>None</td>
<td>None</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>4. NSAID induced aseptic meningitis</td>
<td>None</td>
<td>None</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>5. NSAID induced hypersensitivity pneumonitis</td>
<td>None</td>
<td>None</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

The five groups of pseudo allergic and allergic reactions to aspirin and non-steroidal anti-inflammatory drugs (Taken from Stevenson 2000).
Figure 1.2: The chemical structure of Aspirin and NSAIDs

(Taken from Rang et al, 1999).
Table 1.4: The classification of NSAIDs with respect to their inhibiting effect on cyclo-oxygenase enzymes.

<table>
<thead>
<tr>
<th>Strong Inhibitors *</th>
<th>Weak Inhibitors **</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin (acetylsalicylic acid)</td>
<td>Acetaminophen ***</td>
</tr>
<tr>
<td>Diclofenac (Cataflam Voltaren)</td>
<td>Choline magnesium trisalicylate</td>
</tr>
<tr>
<td>Fenoprofenalcium</td>
<td>Choline salicylate</td>
</tr>
<tr>
<td>Flurbiprofen</td>
<td>Magnesium salicylate (Magan, Mobidin)</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>Propoxyphene HCl (Dolene)</td>
</tr>
<tr>
<td>Indomethacin (indochnron E-R Indocin)</td>
<td>Salicylamide (Lobac)</td>
</tr>
<tr>
<td>Mefenamic acid</td>
<td>Salsalate ***</td>
</tr>
<tr>
<td>Nabumetone (Relafen)</td>
<td>Sodium salicylate</td>
</tr>
<tr>
<td>Naproxen (naprelan, Napron X, Naprosyn)</td>
<td></td>
</tr>
</tbody>
</table>

Non-steroidal Anti-inflammatory Drugs grouped by the degree of cyclo-oxygenase inhibition. * Should be avoided in patients with aspirin sensitivity ** Are tolerated in most patients with aspirin sensitivity. *** Can inhibit cyclo-oxygenase at high doses.
1.3.1.4 Pharmacological side effects of aspirin and NSAIDs

Aspirin is a weak organic acid, with pKa of 3.5 and is relatively insoluble in water (Brody et al, 1998). It is largely unionized in the acid environment of the stomach, and it occurs mostly bound to plasma protein. Aspirin and NSAIDs are mainly effective against pain associated with inflammation or tissue damage because they decrease production of the prostaglandins that sensitise nociceptors to inflammatory mediators. Prostaglandins are not stored within cells, and so their release depends on their biosynthesis. Prostaglandins are formed by regulatory enzyme, known as COX, which catalyses the conversion of arachidonic acid to prostaglandin. PGH$_2$ is subsequently converted to a variety of eicosanoids that include PGE$_2$, PGD$_2$, PGF$_2$, PGI$_2$, and thromboxane (TXA$_2$) (Dubois et al, 1998, Cashman et al, 1995).

Particularly all mammalian cells synthesize prostaglandins; however, the efficacy of inhibitory drugs varies with the tissue. Aspirin and NSAIDs are responsible for nearly a quarter of the adverse drugs reactions reported officially in the UK, and they also feature in the reports of drug related deaths, particularly when used extensively in the elderly for joint disease with fairly large doses and for long periods. The main pharmacological and common side effects are outlined below. (a) The most frequent problem with aspirin is it propensity to cause gastrointestinal disturbances, which lead to bleeding. This is due to the inhibition of gastric mucosal cyclo-oxygenase with consequent loss of the mucosal-protecting action of the prostaglandins. A study done in 200 individuals with normal digestive ability to aspirin showed that most lost 2-6ml of blood per day in the faeces (Rang 1999, May et al, 1999).

In addition the inhibitory effect on platelet cyclo-oxygenase, which decrease platelet aggregation that contributes to bleeding. (b) Skin reactions are the second most
common unwanted reaction. The type of skin condition varies from mild rashes, urticaria, and photosensitivity reactions. (c) Worsening of asthma and polyps in sensitive patients, and salicylism that can occur after repeated ingestion of large doses of aspirin (salicylate). This is a syndrome consisting of tinnitus, vertigo (a sensation of spinning, akin to being drunk) decreased hearing and sometimes also nausea and vomiting (Szczeklik et al, 2000, Feldman et al, 2000).

1.3.1.5 Mechanism of inhibition of cyclooxygenase

In response to injury, infection or inflammation tissue tends to produce cyclooxygenase, which binds to Arachidonic acid (polyunsaturated fatty acid) and prompts the release of inflammatory mediators, in particular histamine, serotonin, platelet activating factor, and the synthesis of prostaglandins, which influence the elasticity of blood vessels, control uterine contractions, direct the functioning of blood platelets that help stop bleeding, and regulate numerous other activities in the body, by sensitising nociceptors to the actions of other mediators. Stimulation of these receptors leads to transmission of pain messages to the brain. Prostaglandins are responsible for producing pain and inflammation in damaged tissues. It has been found that PGE₁ or PGE₂ injected into the peritoneal cavity of mice elicited a stretching response not antagonized by aspirin, they suggested these chemicals could be one of the pain mediators released by noxious stimulation or the final link between the stimulus and activation of pain receptors (Cashman et al, 1995, Wang et al, 1996).

Certain drugs such as aspirin and NSAIDs and corticosteroids counteract the effects of prostaglandins within the body. Arachidonic acid is the predominant fatty acid in most mammalian tissue. It is found in high concentration in most animal fats and so

The mode of action of Aspirin and NSAIDs is due primarily, if not exclusively, to the inhibition of prostaglandin biosynthesis. The worldwide study by pharmacologist Vane in 1970, noted that many forms of tissue injury were followed by the release of prostaglandins. He found that two forms of prostaglandins caused redness and fever, common signs of inflammation. Vane and his co-workers also showed that, by blocking the synthesis of prostaglandins, aspirin prevented blood platelets from aggregating, one of the initial steps in the formation of blood clots. Most NSAIDs inhibit the cyclooxygenase enzyme simply by interfering with the binding of Arachidonic acid (in a reversible manner to a site (Serine residue 506) within the channel through which arachidonic acid must be inserted to be metabolised (Vane 1994,Vane et al 1998). In contrast, aspirin binds rapidly in an irreversible (Serhan and Clish, 2000), competitive manner to the enzyme-binding site and causes conformational change in the active site (Figure1.3), such that Arachidonic acid cannot be converted to PGE2 and PGH2 for the life of the cell (Cronstein et al, 1999, Patrono 1994, Douglas et al, 1993, Gryglewski et al, 2000).

1.3.1.6 Cyclooxygenase iso-forms

The two isoforms are almost identical in structure but have important differences in substrate and inhibitor selectivity and in intracellular location. Aspirin and most NSAIDs inhibit both COX-1 and COX-2, with inhibition predominantly directed at COX-1. Recent studies suggest that the unwanted side effects of aspirin and NSAIDs are due to the inhibition of COX-1, whilst their therapeutic effects are due to inhibition of COX-2 (Picadoc and Valero, 2001).

Aspirin (ASA) is 100 times more effective at disabling COX-1 enzyme than COX-2. The irreversible inactivation of COX by aspirin may last for 10 days or more, leading to numerous adverse effects, including gastrointestinal disturbances such as peptic ulcers. Severe renal bleeding is a less common complication (Cashman et al, 1995). Most acetyl salicylic acid drugs are un-selective inhibitors, however, there are two NSAIDs selective inhibitors of COX-2 (Lanas 2001, Picado and Valero, 2001). Some studies show that ASA is a much weaker inhibitor of prostaglandin formation in vitro; this may be due to the fact that the ability of ASA to inhibit COX is influenced by experimental conditions (Giuliano et al, 2001). This observation has led many researchers to look for alternative mechanisms of action to explain the anti-inflammatory activity of salicylate. Such as checking the abnormality of platelet activation, and leukotrine secretion.
Figure 1.3: The inhibition of prostaglandins production by aspirin and NSAIDs.

(A-B) The production of prostaglandins G and H after the binding of arachidonic acid with cyclooxygenase enzyme, which are in turn converted to a number of bioactive prostanoids (PGE2, PGI2, TXA2). (C-D) the inhibition COX enzymes by Aspirin and NSAIDs.

To date there is no study that explains fully the mechanism and mode of action of aspirin or salicylate. Despite the amount of literature regarding salicylate, particularly the popular theory that salicylate inhibits platelet aggregation, and induces a long lasting functional defect in the platelets, no one has actually explored the effects of these components upon the expression in vitro. Since the response of patient platelets to NSAIDs is similar to the platelet response to IgE-dependent activation, most of the studies found that the lowered platelet activity does not seem to be involved with the increase in lipoxygenase metabolites by inhibition of the cyclo-oxygenase pathway as the cause of oxygen-free radical production (H$_2$O$_2$) (Feldman et al, 2000, Patrono 1994). Others reported that aspirin induced asthma might not be merely the consequence of a cyclo-oxygenase blockade, but as a result of the interaction of PGH$_2$ with its platelet receptor (Evsyukova 1999, Taylor et al, 1996, Durham et al, 1985). Taken together, these observations suggest that in vitro platelet activation is not a reliable indicator of in vivo sensitivity, as the diagnostic test to detect the platelet activation such as chemiluminescence is highly specific but not very sensitive. Other studies suggest that the blockage of COX by aspirin leads the body to use other alternative pathway represented in 5-lipoxygenase (Sanak 2000a, Sanak and Szczeklik, 2000, Kawagishi 2002, Mita 2001). This in turn increases the production of Leukotrienes C$_4$, LTD$_4$ and LTE$_4$, which are able to exert a powerful bronchospastic action (O’sullivan et al, 1995). These studies found that AIA patients excreted significantly more urinary LTE$_4$ than ATA (aspirin tolerant asthma) patients. However, there is little evidence that the increase in LTC$_4$ synthesis occurs as a result of the diversion from the cyclo-oxygenase pathway to 5-lipoxygenase as a response to relatively low doses of aspirin or NSAIDs (Figure 1.4) (Lee 1993, Sanak 2000b, Schiavino 2000, Vives 1998, Israel et al, 1993).
Figure 1.4: Cell mediators released through granules and the two major pathways of arachidonic acid.

(I) Granule release of pre-formed mediators. (II) Lipoxygenase and cyclo-oxygenase pathways and the metabolism of arachidonic acid to produce newly synthesized mediators. (Taken from Roitt 1991).
1.3.2 Natural products (Salicylates in food)

Adverse reactions to food can be divided into four types. The first type, classical food allergy (as in peanut anaphylaxis) where an immediate IgE-mediated reaction is involved. This causes mast cells to degranulate and release histamine, leading to tissue inflammation and swelling. The second type, food intolerance, is not an immune mediated IgE-reaction but may be due to enzyme deficiencies, pharmacological, metabolic, and other mechanism which mimic true food allergy reactions. The third type is due to food components, which lead to toxic reactions. Subsequently a food aversion may result where subjects are convinced that they are allergic to a food but when they are challenged with it, they fail to have any reaction (Bindslev-Jensen 1998, Williams et al, 1989).

Salicylates occur naturally in food. It is estimated that an average daily diet consists between 10mg and 200mg (one tablet of aspirin contains 300mg). A study of subjects taking aspirin showed that salicylic acid is found in higher concentrations in vegetarian serum than non vegetarians as their diets contain higher levels of fruits and vegetables rich in salicylates than non vegetarians (Blacklock 2001). Certain preservatives and food colourings may induce similar symptoms to those aspirin-like drugs induced by in sensitive individuals (Kawane 1994). Food additives and colourings (artificial colouring) such as sodium benzoate, sulphites and tartrazine may elicit an acute flare up reaction of urticaria and rarely gastrointestinal symptoms (Szczeklik 1992, Bindslev-Jensen 1998).

Tartrazine is a yellow dye found in many products including alcoholic and soft drinks, sweets, luncheon meats, preserves, jams, ice cream, coloured baked goods, toothpaste, and mouthwash as some medications. It can induce asthma, and can affect 40% of
aspirin–intolerant asthmatics. Unlike aspirin and NSAIDs, tartrazine does not inhibit cyclooxygenase activity. Instead it is capable of inducing leukotriene release in atopic dermatitis patients with a food intolerance proved by the study done by Worm and his co-workers (Worm et al, 2001). However, there appears to be a significant cross-reacting between responses to aspirin and tartrazine in many aspirin sensitive patients. A study done by Chafee and Settipane 20 years ago showed the similarity to these components in the chemical structures of aspirin and certain azo dyes that cause asthma, i.e. that the chemical structure to tartrazine is similar to that of acetylsalicylic acid. In addition these substances all contained acidic sulphonate (SO₃H) or carboxyl (COOH) group; carboxyl groups are the characteristic molecular feature of the largest class of NSAIDs (Bhatia 2000, Kawane 1994).
Table 1.5: The amount of salicylate content in some foods.

<table>
<thead>
<tr>
<th>Condiments</th>
<th>Low</th>
<th>Moderate</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coriander - fresh</td>
<td>0.2</td>
<td>Chilli - Dry</td>
<td>1.4</td>
</tr>
<tr>
<td>Parsley</td>
<td>0.1</td>
<td>Vanilla- liquid</td>
<td>1.4</td>
</tr>
<tr>
<td>Fruit</td>
<td>Low</td>
<td>Moderate</td>
<td>High</td>
</tr>
<tr>
<td>Apples</td>
<td>0.1</td>
<td>Grapes - fresh</td>
<td>1.9</td>
</tr>
<tr>
<td>Kiwi - fresh</td>
<td>0.3</td>
<td>Melon Cantaloupe</td>
<td>1.5</td>
</tr>
<tr>
<td>Vegetables</td>
<td>Low</td>
<td>Moderate</td>
<td>High</td>
</tr>
<tr>
<td>Olives</td>
<td>0.3</td>
<td>Peppers – Red chilli</td>
<td>1.2</td>
</tr>
<tr>
<td>Nuts and Seed</td>
<td>Low</td>
<td>Moderate</td>
<td>High</td>
</tr>
<tr>
<td>Brazils</td>
<td>0.5</td>
<td>Peanuts Fresh</td>
<td>-1.1</td>
</tr>
<tr>
<td>Walnuts</td>
<td>0.3</td>
<td>Pistachio nuts</td>
<td>-0.6</td>
</tr>
<tr>
<td>Drinks</td>
<td>Low</td>
<td>Moderate</td>
<td>High</td>
</tr>
<tr>
<td>Coffee Beans</td>
<td>0.5</td>
<td>Nescafe Instant</td>
<td>0.6</td>
</tr>
<tr>
<td>Herbal tea Fruit</td>
<td>0.4</td>
<td>Tea Old Chinese</td>
<td>1.9</td>
</tr>
<tr>
<td>Alcohol</td>
<td>Low</td>
<td>Moderate</td>
<td>High</td>
</tr>
<tr>
<td>Beer</td>
<td>0.4</td>
<td>Rum</td>
<td>-1.3</td>
</tr>
<tr>
<td>Vodka</td>
<td>0.0</td>
<td>Wines</td>
<td>-0.9</td>
</tr>
<tr>
<td>Sugars</td>
<td>Low</td>
<td>Moderate</td>
<td>High</td>
</tr>
<tr>
<td>Carob</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The measurements are in mgs /of Salicylate per 100 gms. (1/4lb.) of food or 100 mls. of liquid. Date extracted from “Salicylates in foods”, (Dr Brain Davies LLandough hospital 2000).
1.3.2.1 Mechanism of salicylates

As mentioned earlier salicylic acid was initially extracted from willow bark as an anti-inflammatory agent over 100 years ago. Yet, it has remained the most commonly used drug for relieving pain, fever, and inflammatory symptoms. Aspirin has the ability to prevent myocardial infarction and strokes. Furthermore, it has the ability to reduce the risk of fatal colon cancer (Kenneth 2000). Aspirin has a short half-life in the blood (≈ 20 min), being rapidly metabolized into salicylates (salicylic acid) in vivo, which is the predominant chemical in the plasma and binds strongly to plasma albumin (80-95%) and within 2 or 3 hours little ingestion if any aspirin can be detected in the blood (Kenneth 2000, Feldman 2000).

A study done in healthy subjects taking sodium salicylate showed that these subjects excreted a significantly lower amount of prostaglandin in urine than those not taking sodium salicylate, and their levels of inhibition were comparable to those of patients taking aspirin and indomethacin. This suggests that salicylate may have a direct affect on the inhibition of COX-1 or COX-2 activity. This study and several other reports have made observation in vitro, but the pharmacological relevance in vivo is questionable, because the concentration of salicylate and aspirin (≥ 5mmol/L) that gave these effects on transcriptional and other cellular signalling pathways in vitro are toxic to humans. This data suggests that salicylate inhibits COX metabolism by a mechanism different from direct inhibition of COX activity. Hence, the anti-inflammatory and antineoplastic action of aspirin and salicylate remains a dilemma (Kenneth 2000, Feldman 2000).

Another study on T cells pre-incubation with salicylates such as ASA or Sodium salicylate showed these drugs were able to reduce the capacity of circulating resting T
lymphocytes to adhere to and transmigrate through endothelial cells activated by cytokines, by blocking the integrin conformational changes induced by T-cell activation. This activity might explain the protective effect of ASA against ischemic cardiovascular diseases, which leads to atheroma formation and plaque disruption. Sodium salicylate does not share either the cyclooxygenase inhibition or the ulcerogenic action of ASA, but, like ASA, it reduces adherence and migration of T cells (Carlos and Harlan, 1994). This suggests that the effects of ASA on T-cell adhesion and migration are probably not dependent on the inhibition of cyclooxygenase and prostaglandins metabolism. Several studies suggest that in human lymphocytes aspirin induces alteration in membrane fluidity. This occurs when salicylates enter the plasma membrane and may effects lipid composition; resulting increase in cell membrane permeability and this may be responsible for the observed hemolytic effect of aspirin (Flescher et al, 1995).

1.4 Does sensitivity to aspirin represents a type (I) immediate response?

Several studies considered reactions to aspirin as IgE-mediated. However, a number of studies disagree with this view. The first hypothesis is supported by the similarity of these drugs (aspirin and NSAIDs) reactions and allergic reactions, as similar mediators and amplification systems are involved (Szczeklik and Nizankowska, 2000). Studies have shown the marked elevation of non-specific release of histamine from mast cells or basophils. In addition, the activation of thrombocytes, cytokines and complement (Lee 1993, Vervloet and Durham, 1998, Wedi et al, 2000). However, other studies give at least four reasons against this hypothesis (the adverse reactions to aspirin is IgE-mediated). Firstly, these drugs cause direct histamine release from mast cells. Activated complement components C3a and C5a may also activate mast cells
directly (Abrahamsen et al, 2001). Secondly, specific IgE antibodies to aspirin and other NSAIDs are rarely detectable in the serum of most patients (Ameisen et al, 1985). Thirdly, allergic skin conditions including urticaria and angioedema as well as atopic dermatitis are less clearly defined as true allergic conditions. Hence, chronic urticaria and angioedema are seldom due to a definable IgE mechanism. They are usually a response to a drug, food or food additive (Asero et al, 2002, Stevenson 2000, Wedi et al, 2000). Other investigators indicate that the time to onset of symptoms after oral aspirin intake (45-75min) is more delayed than typically observed in type I allergic responses (Varga et al, 1999, Sczeklik 1997).

True allergic reaction is always accompanied by histamine release and depends on the nature of the allergen. Generally it is characterized by swelling and hive-like breakouts, and sometimes red, raw skin. Most often, skin does not reach true allergic contact dermatitis the first time it is exposed to allergen (Szczechlik and Nizankowska, 2000). In many cases, it takes years and several exposures for sensitivity to develop to a particular allergen (Asero et al, 2002, Stevenson 2000, Wedi et al, 2000). The reaction to aspirin and NSAIDs manifests as immediate cutaneous sensitivity (as urticaria, angioedema, or anaphylaxis). Respiratory reaction to aspirin and NSAIDs begins within a few minutes to hours after ingestion and includes the classic symptoms of asthma (chest tightness, wheeze, and dyspnoea) accompanied by rhinorrhea, conjunctival irritation and flushing of the face and neck, these symptoms may occur in isolation or in any combination with other symptoms. (Schaefer et al, 1999, Vervloet and Durham, 1998).
1.5 Aims

This work will aim to address the following questions:

- Whether sensitivity to aspirin and NSAIDs and salicylates in foods are related effects.
- To investigate and identify the commonality of response between atopic subjects with salicylate sensitivity and non-atopic subjects.
- To demonstrate any underlying role of cytokines in this condition.

Summary of thesis objectives

The primary objectives of this thesis are to:

- Examine the expression of CD63 production in activated basophil cells in vitro by using flow cytometry.
- To investigate whether PGF2α induces displacement of aspirin in plasma protein (albumin) in vitro, by using enzyme-linked immunosorbent assay.
- To study and investigate the release of IL-4 by cells from aspirin sensitive subjects in response to aspirin. To determine the expression of IL-4 mRNA in leucocytes of atopic patients and aspirin sensitive patients following aspirin challenge in vitro by using quantitative RT-PCR.

The secondary objective is to critically evaluate the usefulness of selected investigated cellular and molecular markers as specific diagnostic in vitro tests for predicting individual who are sensitive to aspirin and NSAIDs. In addition to assess the contribution that any finding may have in increasing the understanding of the mechanisms involved in sensitivity to salicylate and NSAIDs.
Chapter Two

The value of up-regulation of CD63 expression in the
diagnosis of AIA and salicylate sensitivity
2.1 Overview

Pseudo-allergic reactions are caused by a variety of drugs such as acetyl salicylic acid (ASA), commonly known as aspirin, and other nonsteroidal anti-inflammatory drugs (NSAIDs). The clinical symptoms caused by these drugs resemble immediate hypersensitivity reactions that consist of urticaria, angioedema and bronchospasm. However, it is well known that aspirin intolerance is not mediated by specific IgE antibodies. No specific in vitro abnormalities, neither humoral (IgE and/or IgG) nor cellular (lymphocytes), have been detected in patients who were reported as sensitive to these drugs (Wedi et al, 2000).

The exact mechanism involved in aspirin sensitivity remains unknown. To date, no in vitro test has been found to be reliable. Also most of these tests are time consuming. Therefore, the goal of this study was to seek reliable procedures for in vitro diagnosis to ASA.

Several studies have concentrated on basophils and their important role in the pathogenesis of atopic diseases such as bronchial asthma, atopic dermatitis, and atopic rhinitis. In allergic subjects, basophils exist in higher numbers and are more activated compared with those that exist in non-atopic control subjects (Monneret et al, 1999, Bochner 2000, Sanz et al, 2001, Crockard and Ennis, 2001b).

Basophils are a minor fraction of blood leukocytes, approximately < 0.2% (0-216 mm$^3$). A number of reports have clarified the potential role of basophil activation in patients who are aspirin sensitivity, which could be used as technique in diagnosing aspirin sensitivity (Sanz et al, 2001).
2.1.1 Basophil

Basophils are bone marrow-derived granulocytes, which share a common stem-cell precursor with eosinophils. They are distinguished by their large purple to blue-black granules in the cytoplasm overlying the nucleus that contains 2-3 lobes (Figure 2.1). These granules are formed in the myeloplast stage and continue to be made during the later maturation stages. The granules have an affinity for basic dyes such as toluidine blue, methylene blue or thiazine dyes. Basophils are thought to have a function similar to that of mast cells, but their relationship is controversial (MacGlashan et al, 2002). Both cells participate in a similar manner in acute and delayed allergic reactions. They have similar granules and morphological structures and each contain histamine and heparin. However, mast cells rarely enter the blood. They are predominant in the connective tissue of various organs, bone marrow, and the mucosal areas of serous membranes (Pallister 1998, Moneret-Vautrin et al, 1999, Abuaf et al, 1999, Knol et al, 1990, Sanz et al, 2001).

Granules in basophils vary in size from 0.1-0.3 μm in diameter; they are coarse and unevenly distributed and are water-soluble. In cells that are poorly fixed during staining, the centre of the granules may disappear or the entire granule may be washed away, leaving a small colourless cytoplasmic area. Maturation of basophils takes place over seven days. These cells circulate for a few hours in blood, and then migrate into skin, mucosa, and other serosal areas (Pallister 1998) (Figure 2.2).

Basophils are important effector cells of inflammatory reactions, and widely used in allergy diagnosis. Human basophil and mast cells are central to the pathogenesis of chronic allergic disease. In contrast with eosinophils they are the only cells that synthesise histamine and express plasma membrane receptors (FceRI) that bind with
high affinity to IgE at the Fce portion (Cozon et al, 1999, Tsang et al, 2000, Bühring et al, 1999, Sihra et al, 1996). Their role in allergic reactions is directly related to their content of pharmacologically active substances such as histamine, heparin and platelet activating factor. *In vitro*, basophils from sensitized subjects degranulate in response to allergens. Chan and Yoffey reported that the numbers of basophils in the blood and bone marrow increase with repeated antigenic challenge (Hall and Malia, 1991, Pallister 1998). These cells produce various active mediators, which after cell stimulation can be released into the extra-cellular space. Typically, not all basophils are seen to be degranulating. In fact, a spectrum of morphologic changes is usually seen after activation, some cells remaining relatively unchanged, and others partially or fully deregulated (Bochner et al, 1991).

2.1.2 Basophils response in disease

The response of Basophil to allergens remains poorly defined because no specific marker exists to detect basophils in tissue or in blood. In addition the low numbers of basophils in whole blood (0.5%-2%) limit their availability for study. Basophils are motile and are attracted by chemotaxins such as complement and products of activated lymphocytes. The phagocytocytic power of basophils is weak but they have considerable pinocytic capability in transporting exogenous compounds to cytoplasmic granules (Toba et al, 1999, Kepley et al, 1994, Miroli et al, 1986). So far, only a few markers that show specificity can be expressed by human basophils, including (IgE, CD45, CD18, and CD63) (Cozon et al, 1999, Sainte-Laudy et al, 2002, Bühring et al, 1999).
In this study, measurement of basophil activation was performed by monitoring changes on the basophil surface after activating the cells \textit{in vitro} with aspirin (L- acetyl salicylic acid).

\textbf{Figure 2.1: Normal basophil in peripheral blood smear.}

Arrow indicates a basophil with dark blue granules, which often obscure their segmented nucleus (Taken from Harmening 1992)
The arrows indicate the basophil and mast cell granules, which can be seen in the ultra-structure above as dense round and oval structures. These granules contain sulfated glycoaminoglycans. In normal basophils, heparin is the predominant constituent. Basophil granules are the major source of circulating histamine. Trypsin and kallikrein are also stored in the granules (Taken from Harmening 1992).
2.1.3 CD63

The diagnosis of drug allergy is mainly based upon a detailed clinical history, positive skin tests and detection of specific IgE. Occasionally a histamine release test (HR) is performed, however, the diagnostic relevance of this test remains controversial. Many reports suggest that diagnosis of drug allergy using HR is of little benefit due to the short life of histamine in the blood cycle, time consuming procedure and the cost of reagents (Monneret et al, 1999).

Cluster differentiation (CD) is detected usually with mouse monoclonal antibodies raised against a human antigen (a particular site in the cell surface). These antigens represent a vast number of entities in the cell, ranging from adhesion molecules and complement fragment to highly specialized enzymes. Each antibody recognizes only one binding site (its specific antigen) i.e. an antibody would only bind the antigen it was raised against but would not react with any immunologically different antigens. However, if the antigen is part of a big complex it is possible that different (CDs) molecules would target the same complex, but at different binding sites (antigens) of that complex. Identification of a certain cell phenotype is simply based on the presence or absence of certain antigens in those particular cells. Therefore, an indication of differentiation could be simply assessed by difference in particular CD expression pre and post differentiation induction by a certain drug (allergen). In an attempt to find a more sensitive and specific test for the diagnosis of salicylate sensitivity, CD63 expression on basophils was studied (Taylor et al, 1996, Sainte-Laudy et al, 1998, Moneret-Vautrin et al, 1999, Abuaf et al, 1999).

CD63 is granule protein (53-kDa lysosomal membrane protein) first found in platelet lysosomal granules, monocytes and macrophages. It is widely expressed on the
surface and in the cytoplasm of various hematopoietic (monocytes, macrophages) and non-hematopoietic cells, such as (endothelium, fibroblasts, osteoclasts, and smooth muscle) after platelets activation. (Taylor et al, 1996, Moneret-Vautrin et al, 1999, Abuaf et al, 1999, Knol et al, 1990, Sanz et al, 2001).

In resting basophils, CD63 is located on the intracytoplasmic granule membrane. After activation of basophil and cell degranulation due to the fusion of granule membrane with the plasma membrane, CD63 is translocated as a result of basophil activation and its expression with a high density on the cell surface is increased. There is a strong positive correlation between the release of histamine and an increase in CD63 expression (Monneret et al, 1999, Moneret-Vautrin et al, 1999).

CD63 is considered to be the most useful marker of basophil activation (Crockard and Ennis, 2001a). The activation of basophilic granulocytes can be quantified via measurement of mediators (stored or de novo synthesised), thus by using CD63 cell surface expression after activation, release of mediators following activation can be detected (Knol et al, 1991). A definitive biological function for CD63 is not known but many suggest that it may participate in signal transduction processes between platelets and other cells. At present CD63 represents a useful marker for monitoring events involved in allergic reaction to food and drugs as well as allergy to specific allergens such as venom allergy (Sainte-Lauy et al, 2000) latex and house dust mites (Cozon et al, 1999, Abuaf et al, 1999, Taylor et al, Moneret-Vautrin et al, 1999, Crockard and Ennis, 2001a).
2.1.4 Aims

- To investigate and analyze changes in CD63 expression in basophils activated by acetyl salicylic acid.

- To evaluate whether changes in CD63 expression could be used as a predictive test for aspirin sensitivity.
2.2 Materials and Methods

2.2.1 Use of Flow cytometry for CD63 detection

The diagnosis of allergic disease requires objective tests to confirm it. In allergic disease, a skin test is commonly used. However this is not always an accurate test since in some cases individuals may react and lead to false positive results. Indeed both oral challenge and inhaled challenge, may cause anaphylactic reactions in some patients. Several papers described the potential use of flow cytometry “Fluorescence Activated Cell Sorting” (FACS) in the diagnosis of allergic diseases, concentrated mostly on allergen induced basophil activation. In addition, lymphomas and leukaemias are intensively studied for surface markers of diagnostic and prognostic value (Sanz et al, 2001). Therefore, inclusion of FACS based assays such as immunophenotyping by Cluster differentiation (CDs) surface markers and cell cycle analysis could shed a clear prospective on the outcome of the differentiation processes. It has been the method of choice for monitoring CD4 lymphocyte levels in the blood of AIDS patients (Sainte-Laudy et al, 1998), CD4 and CD8 T cells as markers in atopic and nonatopic child hood asthma (Gemou-Engesaeth et al, 2002) and CD63 as marker for Dermatophagoides pteronyssinus allergic (Sanz et al, 2001).

The main concept of (FACS) technology relies on detection of emitted light characteristic to individual cells. Briefly, the technique allows the analysis and simultaneous measurement of multiple physical characteristics of single cells. These measurements are made while cells pass single file through a laser beam at a rate of 500-4000 cells per second in a fluid stream. Cells may be stained by a fluorochromic dye, exciting them, giving the ability to emit light at a certain wavelength. Each cell
scatters some of the laser light, and also emits fluorescent light excited by the laser, which can be detected by the flow.

- **Forward-scatter intensity (FSC)**, where light is diffracted at a low angle (1-10 degrees). The main proportion is to cell diameter (size).
- **Side-scatter (90 degree) intensity (SSC)**, which measures the quantity of granular structures within the cells (complexity).
- **Fluorescence intensities** are measured at several different wavelengths (FL1 - FITC), (FL2-PE), and (FL3-PerCp).

Fluorescent probes are used to report the quantities of specific components of the cells. The fluorescent antibodies are often used to report the densities of specific surface receptors and the number of binding sites, and thus to distinguish subpopulations of differential cell types. Finally, the cells flow past the detector point and are illuminated, the probes fluoresce, and the emitted light is detected and converted into electronic signals containing cells of interest. Under the computer control, the data will be recorded for thousands of cells per sample, and displays the data graphically. Collectively, the light patterns are translated to data concerning cell size, shape composition, granulation, convolution, vacuolization, nucleic acids content, etc (Figure 2.3).

**2.2.1.1 Cell surface marker detection**

In flow cytometric analysis, where cells are identified by the possession (or lack) of certain cellular markers, known as Clusters differentiation (CD). They are proteins on the cell membrane that can be detected with antibodies coupled with fluorescent dyes. Different CDs are expressed at different stages. Some present early in development while others do not appear until much later. Some proteins may appear, then
disappear, only to reappear at a later stage of development. This unique expression of proteins enables them to be used as markers of both cell lineage and maturation stages. In this study, CD63 will be used to investigate the activation of basophil upon treatment with ASA (acetyl salicylic acid) with List of CDs used in this work (Table-2).

Table 2.1: CDs investigated in this study.

<table>
<thead>
<tr>
<th>CD</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD63</td>
<td>Activated platelets, Monocytes, Macrophages, and activated basophils</td>
</tr>
<tr>
<td>CD14</td>
<td>Human Monocytes, Macrophage antigen</td>
</tr>
</tbody>
</table>

Antibodies reagents were purchased from Sigma and were stored at 4°C and protected from light, except for anti-human IgE, which was stored at –20°C.

FACS™ lysing solution (Becton Dickinson) was used for lysing whole blood after immunofluorescence staining and was kept at 4°C until use.
Figure 2.3: Flow cytometry structure.

Two detectors detect the light scatter and three photomultiplier tubes detect the fluorescent signals. The band pass filters are set up for optimal detection with PharMingen’s fluorochromes: FITC, PE, Cy-Chrome and Becton Dickinson’s Per-CP (Taken from Technical Protocols, Pharmingen.Becton Dicksons).
2.2.2 Patients

74 subject patients and normal volunteers, aged from 22 to 69 years were evaluated. Patients were investigated at Llandough Hospital, and UWIC (University of Wales Institute, Cardiff). The initial examination included a positive skin test (prick-test), which was performed on most of the patients against grass pollen, nuts and pets (cat). They were divided into 4 groups according to their diagnosis. 11 non-atopic (control), 24 non-atopic aspirin sensitivity, 31 atopic aspirin and 8 atopic non-aspirin sensitive. The history of the patients and any history of adverse reactions with Aspirin or any salicylate products were noted.

Indication for Aspirin and salicylate sensitivity:
- Redness of the face and upper chest.
- Polyposis.
- Rash after ingestion of any of these drugs (Aspirin - NSAIDs).
- Asthma.
- Angiodema.

2.2.3 Skin Tests (Prick Test)

Skin prick testing was performed in order to determine the atopic status of the subjects included in the study. This test is the most widely used, least expensive, and the easiest technique for evaluating sensitivity to allergen. The test simply produces an allergic reaction on a small area of skin by intentionally exposing the patient to a small amount of an antigen. The test was performed on the volar surface of the forearm. The skin must be free of lesions, cleaned with alcohol and air-dried. A drop of the testing antigen (50 µl) was placed on the skin. Small drops of saline and histamine (50µl) were used as negative and positive controls respectively. The
location of each antigen was marked previously with pen. A lancet needle was used to puncture the superficial layers of the skin without drawing any blood. The test was completed in 15-20 minutes. If any weal reaction occurred before this time, the test site was wiped free of antigen to reduce any unwanted reaction (Booth 1997). The skin test was carried out by Sharon Rolf at UWIC.

2.2.4 Blood samples

Blood specimens were collected from patients and volunteers using vacutainer tubes containing lithium heparin anticoagulant. The basophil activation test was carried out within three hours of the blood being collected, and a few samples were collected the next day. There was no remarkable difference between the blood samples processed after 45min and blood kept for 24h at 4 °C. This concurs with the outcome of several reports. (Moneret-Vautrin et al, 1999, Sanz et al, 2001).

2.2.5 Basophil activation

Several dilutions of Lysine-acetyl salicylic acid (aspirin) were used as follows; 18mg/ml, 9mg/ml 3.6mg/ml, 1.8mg/ml, 0.36mg/ml, and 0.18mg/ml. For each sample 100µl of heparinised whole blood was placed in a labelled plastic FACS tube, mixed with 10µl of L-ASA in order to demonstrate any potential non-specific activation. 100µl of whole blood treated with 10µl of PBS was used as a negative control and 100µl whole blood with 10µl of FMLP (chemotactic peptide formyl-methionyl-leucyl-phenylalanine) was used as a positive control. FMLP is known to increase the activation of basophils (Knol et al, 1991). Samples were then incubated for 30 minutes at 37°C. In a second experiment, grass pollen was used as an allergen and a similar series of tubes with 100µl of whole blood and 10µl of grass pollen dilutions, used with the same negative (PBS) and positive (FMLP) controls as before. Blood
samples were collected from 4 subjects who were sensitive to grass pollen were used in this part. The procedure was performed in (section 2.4.4 - 2.4.5).

2.2.5.1 Labelling of cells

Prepared samples were incubated with 10μl of each labelling reagents with a total of 30μl of three monoclonal anti-bodies, labelled with specific fluorochromes for 15 minutes, in the dark, at room temperature. These labels were polyclonal fluorescein isothiocyanate (FITC), conjugated goat antibodies to human IgE, phycoerythrin (PE) conjugated anti-human CD63, and Per-CP conjugated anti-human CD14, with their respective isotype controls. Erythrocytes were lysed by the addition of 2ml of RBC lysis solution to the tubes and left in the dark, at room temperature for a further 10 minutes. Samples were centrifuged at 2000 rpm for 5 minutes. The supernatant fluid, mainly consisting of RBCs was removed. Cells were washed twice in PBS and centrifuged for 5 minutes at 2000 rpm. Finally, cells were re-suspended in 500μl PBS and analysed. Batched cells were preserved in 2% paraformaldehyde and stored from light at 4°C for a maximum of 3 days. The cells were analysed using Becton and Dickinson FAC scanner. Cells were analysed according to their size and cellular granularity by measuring their forward and side scattered light respectively. At least 1000 cells were processed through FL1, FL2 and FL3 channels in addition to forward and side scatters (Figure 2.4). At the beginning of each acquisition, proper settings were achieved by adjusting parameters such as compensation and channel threshold.

2.2.6 Purification of basophils

Basophils play an important role in the allergic reaction, particularly, in severe asthma, where reports detected their presence in the airways of subjects dying from asthma (Tsang et al, 2000). However, their low number in the blood circulation
<0.2% (0-216 per mm³), limits their use in studies. Several methods have been proposed to enrich the basophil concentration, and isolate them from peripheral blood.

a) Positive immune selection, using IgE and either anti-IgE antibody or antigen. The disadvantages of this method are that other cells may be purified too; such eosinophils and B-lymphocytes are required for low temperatures (Kepley et al, 1994).

b) Negative selection, with antibodies that selectively recognised by other cells such as T lymphocytes (CD2), B-lymphocytes (CD19) and monocytes (CD14) (Mul et al, 1992, Gibbs et al, 1996, Tsang et al, 2000).

c) Gradient centrifugation basophils without using immunological reagents, but purification of cells can be problematical for a number of reasons. Firstly, the isolation procedure used is time consuming and possibly cell damaging, as it usually involves successive density gradients centrifugation steps and repeated washings in a variety of suspending media (Kepley et al, 1994).

d) In this study, basophils were separated from lithium heparin anticoagulated peripheral blood using Ficoll-paque preparation.
2.2.6.1 Preparation of Ficoll-Hypaque solutions

Two Solutions of different densities were prepared:

Solution I: (Ficoll-Hypaque with density of 1.077g/ml). It consists of 3.2 Ficoll and 5.25g Hypaque (Sodium diatrizoate) made up to 50ml with distilled water giving a final density of 1.077g/ml.

Solution II: (Ficoll-Hypaque with density of 1.114g/ml). It consists of 8.182g Ficoll and 15.452g Hypaque that are mixed together and made up to 100ml with distilled water giving final density of 1.114g/ml. The ficoll-pique was placed in the fridge and filtered before use.

Eight of Ficoll-Hypaque of 1.114g/ml followed by 2ml spgr 1.077g/ml was layered into universal tubes followed by a layer of 8ml-tested blood.

The tubes were centrifuged for 35min at room temperature at 450xg (1800rpm). After centrifugation, different layers were observed containing the different populations of separated cells. The supernatant fluid was discarded. The Ficoll-pique layer, inter-phase layer and the buffy coat layer were aspirated using a plastic pipette. The layers were then transferred to separate clean plastic universal tubes and washed twice with PBS, before centrifugation for 15min at 300xg. Pelleted cells were gently re-suspended in PBS, centrifuged and the supernatant fluid discarded. After washing, cells were re-suspended in 2ml 0.5% PBS to achieve a final concentration of 5 x 10^6 cells/ml, ready for immediate use, or were fixed in 4% paraformaldehyde, for use the next day (Miroli et al, 1986). The total number of cells following the separation was determined by counting cell numbers using Coulter® MicroDiff to achieve a yield of 90% purity.
2.2.7 Data acquisition

At least 1000 cells (events) were scanned for the expression of CD63. Adjustments of the proper acquisition setting were made for every sample. Dot plot settings were adjusted for each plot and where necessary, a polygonal region was drawn. The accuracy of the population acquired was assured by gating out unwanted leukocytes, dead cells and debris (Figure 2.4). At the end of each acquisition, analysed using the WinMDI (Department of Haematology, UWCM) software program, a comparison of dot plot results analyzed using the FACS and WinMDI, showed no difference between the values obtained. Results of CD63 expression were expressed as the Mean Fluorescence value.

2.2.8 Statistical analysis

Results were reported as the mean value ± Standard Deviation (SD). T-test was performed, where applicable. All calculations were performed on Minitab release version 12.
2.3 Results

2.3.1 Effects of L-ASA in CD63 expression on activated basophils

Positive skin test results as follows correlate with the history of the studied subject, 11 non-atopic control, 24 non-atopic aspirin sensitivity, 31 atopic aspirin and 8 atopic non-aspirin sensitive.

Though the symptoms of aspirin sensitivity are similar to those produced by allergic or anaphylactic reactions, the mechanism of aspirin sensitive asthma is not clear. Most immunologic tests, including complement activation and histamine release, have been applied to aspirin sensitive subjects, with negative or equivocal results. Recently, most *in vitro* tests are focused on basophils, due to their important role in atopic diseases. Nevertheless, the low number of these cells in the circulating blood limits their use in these experiments. To detect the activation of basophils we measured the expression changes of CD63 as a specific marker on the basophils surface, after activation. The results are expressed as percent change in the mean fluorescence intensity of total basophils population.

Purification of basophils:

As mentioned previously, due to the low number of basophils in the blood circulation, this study purified basophils using the “Ficoll gradient technique” to increase the specificity of the results. The number of activated basophil with L-ASA after purification was similar to those before purification. As this technique was time consuming, and no improvement was detected after the analysis, this step was excluded in subsequent experiments (results are not shown).
Basophils response to FMLP:

Samples showed a wide range of response to FMLP. Most of the tested subjects showed a response to FMLP, as it activated the basophils, and it increased their expression of CD63. In most tested groups, the number of cells activated by FMLP was less than those cells activated by L-ASA in sensitive individuals (Figure 2.5).

Basophil response to PBS:

The baseline for the expression of CD63 after stimulation of cells with PBS was similar in most subjects tested among the atopic non-aspirin sensitive and healthy volunteers. In some patients (36% of the atopic aspirin sensitive group and 19% of the non-atopic aspirin sensitive group), the results were high. Since this could be due to the fact that these patients had naturally activated basophils.

Activated number of basophils:

There were significant differences in the specific fluorescence intensity emitted by basophil expressed CD63 among the four groups (Fig 2.5). In the control group (A), the intensity of CD63 and the number of activated basophils were low. In the atopic non-aspirin sensitive group (B), the result was similar to group (A), 10% of the subjects treated with L-ASA showed rose, but still low CD63 expression. Group (C) showed high CD63 intensity and a high number of activated basophils; this group was non-atopic aspirin sensitive.
Figure 2.4 Image captures from WinMDI software showing the gated activated basophil (positive cells) which labeled according to different fluorescent FL1= anti-IgE FITC against FL2= CD63PE on three groups: (A), control (healthy volunteers) (B) atopic non aspirin sensitive (C) atopic / non atopic aspirin subject. The activated basophils shift to the upper right is clearly observed after L-acetylsalicylic acid challenge in the aspirin sensitive individual.
Figure 2.5: Histograms for the expression of CD63 fluorescence intensity detected on basophils after the samples were treated with L-acetyl salicylic acid (L-ASA). (A) Control (healthy volunteers). (B) Atopic non-aspirin sensitive subject. (C) Non-atopic aspirin sensitive subjects.
Aspirin sensitive groups:

Upon *in vitro* stimulation with aspirin, there was a significantly greater expression of activated basophils 50-76% in aspirin-sensitive groups (atopic or non-atopic) (Figure 2.4-C). In contrast, there was a significantly reduced expression of activated basophils in atopic non-aspirin subjects and healthy volunteer subjects, a 15-35% reduction, and 2-10% reduction respectively (Figure 2.4-A, B).

Nine patients out of 57 of the aspirin sensitive groups (atopic or non-atopic) showed high number of activated basophil but low fluorescence intensity of CD63. In contrast, 17 of these patients had a low number of basophils with high fluorescence density of CD63 expression.

The activation of basophils in relation to aspirin dose:

It was noteworthy that there was a dose concentration dependence of ASA. It was noticed that a high concentration of L-ASA (1.8mg/ml) was able to suppress the expression of CD63, which was contradictory to the effect seen at low concentrations (Figure 2.6-2.7). High doses of L-ASA (18mg/ml) were found to significantly induce CD63 expression in all patients tested. At 1.8mg/ml of L-ASA the expression of CD63 reached its maximum in all the tested groups (Figure 2.7), although, this expression varies between the four groups, even within the same group the expression of CD63 and the number of activated basophil cells varied per subject.
Figure 2.6: The effects of L-ASA on expression of CD63 on the 4 studied groups.

Fluorescence intensity of CD63 % in the four groups studied. **nonA:** non atopic or healthy volunteers. **nonAs:** non atopic aspirin sensitive. **At/As:** atopic aspirin sensitive. **At/nonAs:** atopic non-aspirin sensitive. The non aspirin sensitive groups (nonA & At/nonAs) showed low fluorescence intensity of CD63 expression in comparison with the other two groups who are aspirin sensitive (At/As & non/As). Bars represent the mean ± SEM of 71 subjects.
The effect of L-ASA on the four studied groups response: non A: non atopic or healthy volunteers. non As: non atopic aspirin sensitive. At/As: atopic aspirin sensitive. At/nonAs: atopic non-aspirin sensitive. Aspirin sensitive groups (either atopic or non atopic) showed a higher number of activated basophils compared with healthy volunteer (nonA & At/nonAs. Bars represent the mean ± SEM of the 71 subjects.
2.3.2 Effect of L-ASA on grass pollen sensitive patients

To investigate whether the expression of CD63 was specific for aspirin sensitivity, or whether its expression can be detected in other allergies, the effects of L-ASA on another allergic group was investigated. The selected group was sensitive to grass pollen. In order to confirm that the selected subjects were grass pollen sensitive, skin prick tests were carried out.

The test involved applying a drop of grass pollen antigen (50μl) onto skin, followed by a superficial skin prick. A positive reaction was defined by an immediate skin weal of at least 3mm² in diameter. Histamine hydrochloride was used as positive control, and NaCl₂ 0.9% as a negative control.

Basophils activation was performed as described previously in section 2.44 and 2.4.5. CD63 may be highly expressed in other allergies to verify this in the selected group; preliminary experiment was ran on two subjects who were grass pollen sensitivity. Figure 2.8 shows the results of CD63 expression after stimulation of the basophils with grass pollen antigen.

These results show that the patient who is grass pollen sensitive had high basophilic expression of CD63, when the cells were activated by grass pollen, but not when activated with L-acetyl salicylic acid. The effect of L-ASA alone was investigated in all subjects in the grass pollen group. There was no increase in the basophils expression of CD63 in grass pollen patients when their blood was stimulated by L-ASA.
Figure 2.8: The effect of different allergens on basophil expression of CD63

The number of activated basophil expression CD63 after the cells were treated with specific stimuli. L-ASA: indicates cells that were stimulated by aspisol (L-ASA). Grass pollen: indicates the cells were stimulated by grass pollen allergen. Bars represent the mean ± SEM of the 2 subjects.
Figure (2.9): The effects of L-ASA on different subject groups.

Each bar chart representing the expression of CD63 in the four studied groups after treatment with different concentration of L-ASA. (A.AS) is atopic aspirin sensitive, (A.G.P) is atopic grass pollen sensitive, (nA.As) non-atopic –aspirin sensitive, (control) healthy volunteers. The values represent mean ± SEM, which show significant difference between the four studied groups.
Table 2.2: The mean expression of CD63 in 4 studied groups

<table>
<thead>
<tr>
<th></th>
<th>Healthy volunteers</th>
<th>Atopic aspirin sensitive</th>
<th>Non-Atopic aspirin sensitive</th>
<th>Atopic / non aspirin sensitive</th>
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<tr>
<td>CD63 ± SE</td>
<td>PBS</td>
<td>18mg/ml L-ASA</td>
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<td>232 ± 6.8</td>
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Table shows the means ± SE value of the CD63 expression in the 4 tested groups after activation of their samples with 3 different dilutions of L-ASA, and one dilution of PBS.
As illustrated in Table 2.2 above, the first group, the healthy volunteers (controls), no significant difference in the expression of CD63 was observed, after the treatment with L-ASA. In fact, treated (L-ASA) and untreated (PBS) samples continue to give a slight difference in expression of CD63 (P=0.481), yet this difference was not that significant. On the other hand, the atopic aspirin sensitive group showed a clear difference between treated and untreated samples. The expression of CD63 was significantly increased in treated samples (L-ASA-1.8mg/ml) as compared with untreated (P=0.002).

The non-atopic aspirin sensitive group, showed statistically significant differences in CD63 expression (P<0.05) in treated samples with L-ASA. The difference was significant at all concentrations (L-ASA), compared with the three other groups. This group showed high expression of CD63 in all concentrations of L-ASA; except for 18mg/ml the expression was obviously reduced.

In atopic non-aspirin sensitive group, there was a significant difference between treated and untreated samples (P=0.027).
2.4 Discussion

The diagnosis of ASA intolerance is based mainly on clinical history manifestation and verification by potentially harmful challenge tests. Skin testing and IgE antibodies are not suitable diagnostic procedures. Other tests such as histamine release by platelet activating factor, determination of platelet hydrogen peroxide metabolism and urine LTE₄ detection have not found a place in routine diagnostic tests for this condition. There is an increasing interest but limited experience in the determination of LT release in the supernatants of resting and challenged leucocytes with NSAIDs in sensitive subjects. However, the limited sensitivity of the test in ASA > 66% in many studies as well as the necessity for fresh isolated leukocytes from untreated patients are still the major disadvantages that reduce the use of this technique.

Flow cytometry is an analytic technique suitable to quantify the expression of cellular membrane markers, such as CD63. It was used in this study to analyse changes in human basophil surface during stimulus-induced degranulation. The triple - labelling fluorescence allows selection, first of the basophil population with monoclonal antibodies marked against high affinity IgE receptor to gate the lymphocytes population, and then of activated basophils with monoclonal antibodies directed against CD63.

In several studies, anti-CD45 has been used as an additional marker (Monneret et al, 1999, Moneret-Vautrin et al, 1999). In this work, CD45 was excluded from the experiment, as it did not enhance the results. Anti-CD14 was used, which is a specific marker for monocytes, to locate the area of monocyte population, and made it easy to locate the area of interest granulocytes (basophils), which improved the results. To locate the activated basophils, the area of monocytes population was determined by
using CD14 as an indicator for these cells, and lymphocytes were determined by using anti-IgE. This enabled gating of population of activated basophils. The number of activated basophils per sample expressing CD63 ranged from 300-450 cells. This study agrees with (Monneret et al, 1999, Abuaf et al, 1999), in that CD63 expression was up regulated in response to allergy. Aspirin used as allergen in this study lead to the expression of CD63 marker on the membrane surface, presented at a high density, in most of the aspirin sensitive patients. In general, the four groups showed high expression of CD63 after activated the cells with FMLP for 30 min. The result agrees with previously published reports, that FMLP is a strong stimulator (Knol et al, 1991).

In aspirin sensitive patients, the results showed a significant increase in the percentage of activated basophils, after treating the cells with L-ASA, compared with non-treated cells. L-ASA has an effect on the expression of CD63. At high doses of L-ASA 18mg/ml, the expression of CD63 was suppressed, while at low doses, expression was enhanced until it reached its optimum at 1.8mg/ml of L-ASA, in most of the tested patients. Furthermore, these findings comply with previous studies where it was noticed that high doses suppress the expression of CD63 (Monneret et al, 1999, Abuaf et al, 1999). There is no explanation for the overall decrease in expression of CD63 at 18mg/ml dilution of L-ASA. This raises the possibility that high concentrations of L-ASA be toxic to the cells, leads to down regulation of CD63 expression.

Although, optimal expression of CD63 was detected at 1.8mg/ml. It cannot be specified as immunologically specific standard concentration for optimal expression of CD63, since other concentrations gave high expression of CD63 in some patients such as 3.6mg/ml.
The expression of CD63 makes the measurement of activated basophils possible. The increase in expression after stimulation with L-ASA varies from subject to subject. Several subjects showed a low number of activated basophils (events) whilst at the same time they expressed high fluorescence intensity of CD63.

The presence of high CD63 intensity in 17 patients with low basophil number, together with low intensity in 9 patients with high basophil number may be explained in several ways. Firstly, it could be related to the difference in CD63 receptor number. In the first group, the increased in CD63 expression might be directly related to the increase in the numbers of CD63 receptors on the surface of basophils. Secondly, this probably refers to the patients and the amount of CD63 presents in their cells, like the amount of histamine in the basophils (Knol et al, 1991).

To determine whether CD63 expression was specific for aspirin aggravated allergies, the study was repeated on patients who are grass pollen sensitive (section 2.7.2). The results indicate that allergies other than aspirin can cause elevated expression of CD63. It must therefore be concluded that CD63 expression is not specific for aspirin sensitivity but can also be used to detect allergies, when aggravated by the causative agent. Thus, CD63 detection by flow cytometry seems to be a reliable method for the detection of activated basophil cells by drugs (ASA). Furthermore, flow cytometry may represent a fast and sensitive method for diagnosis of drug sensitivity using basophil activation.
Chapter Three:

An investigation into the use of serum prostaglandin F$_2$ alpha (PGF$_2\alpha$) released from bound serum protein as a diagnostic marker for AIA and salicylate sensitivity
Chapter Three

An investigation into the use of serum prostaglandin F$_2$alpha (PGF$_2$α) released from bound serum protein as a diagnostic marker for AIA and salicylate sensitivity
3.1 Overview

When allergens cross-link IgE bound on mast cells, they become activated and degranulate within seconds, and result in a series of chemical events. Calcium (Ca) ions enter the cells, and the intracellular granules and their preformed mediators are released. These mediators include, histamine, a short-lived vasoactive amine, heparin proteoglycan, and other chemotactic factors. This immediate allergic reaction is followed by the recruitment of other effector cells such as, basophils, eosinophils, and lymphocytes.

New mediators can be generated from arachidonic acid metabolism these are freshly synthesised and released over a longer time-scale from two different pathways, the cyclooxygenase and lipoxygenase pathways. They are known as secondary mediators or SRS (slow reacting substance) that includes “prostanoids” such as prostaglandins and thromboxane, formed by the cyclo-oxygenation of arachidonic acid.

The end product of arachidonic acid metabolism differs in different cells. For instance, in platelets, the pathway leads to thromboxane A₂ synthesis, in vascular endothelium it leads to prostacyclin synthesis and in macrophages it leads mainly to synthesis of prostaglandin E₂. Other cells in particular mast cells synthesis PGD₂ (Cashman et al, 1995). The classification of prostanoid (refers to prostaglandins (PGs) and thromboxanes (TX) has been reported by Coleman 1993. Five main prostanoid receptors have been defined, one for each prostanoid, PGD₂, PGF₂α, PGI₂, TXA₂ and PGE₂, termed DP-, FP-, IP-, TP- and EP-receptors, respectively (Cashman et al, 1995, Zeldin 2002).
3.1.1 The main function of prostaglandins

Prostaglandins produce a wide range of effects on the body, including causing pain and inflammation in damaged tissue. Although they do not cause pain or local edema themselves, they strongly enhance the pain producing effects of other agents such as histamine, 5-HT or bradykinin (Copeland et al, 1994).

In healthy individuals, PGs mediate a range of normal biological functions such as gastric protection, vascular and renal homeostasis, embryo implantation and labour, regulation of sleep wake cycle, as well as regulating body temperature and inflammation (Table 3-1).

3.1.1.1 The inflammatory response mediated by prostaglandin

The inflammatory process is a defensive mechanism that protects the body from noxious stimuli and is always accompanied by the release of prostaglandins, which present at inflammatory sites. PGE\textsubscript{2} is the most predominant product in acute and chronic inflammation; this is responsible for producing fever, inhibition of T cell proliferation, as well as inhibition macrophage activation. PGE\textsubscript{2} in particular, modulates oedema and pain (Curtis-prior 1988, Dubois et al, 1998).
Table 3.1: The effects of some prostaglandins

<table>
<thead>
<tr>
<th>Type</th>
<th>Effect</th>
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<tr>
<td>PGA₁</td>
<td>Lowers blood pressure</td>
</tr>
<tr>
<td></td>
<td>May protect against peptic ulcer</td>
</tr>
<tr>
<td>PGD₂</td>
<td>Causes inflammation</td>
</tr>
<tr>
<td>PGE₁</td>
<td>Stimulates contractions of the uterus</td>
</tr>
<tr>
<td></td>
<td>Lowers blood pressure</td>
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<td>Reduces stickiness of platelets in blood</td>
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<tr>
<td>PGE₂</td>
<td>Causes inflammation</td>
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<tr>
<td></td>
<td>Widens airways</td>
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<td></td>
<td>Increases stickiness of platelets in blood</td>
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<td></td>
<td>Stimulates contractions of the uterus</td>
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<tr>
<td></td>
<td>Protects against peptic ulcer</td>
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<tr>
<td>PGF₂</td>
<td>Stimulates contractions of the uterus</td>
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<td></td>
<td>Narrows airways</td>
</tr>
<tr>
<td>PGG₂</td>
<td>Causes inflammation</td>
</tr>
<tr>
<td>PGI₂</td>
<td>Reduces stickiness of platelets in blood</td>
</tr>
</tbody>
</table>

Different prostaglandins produced by the tissue, and their function
(Taken from Pathologic Basis of disease, Cotran et al, 1999).
Prostaglandins PGF$_2$$\alpha$ and PGE$_2$ are known to modulate airway calibre. PGE$_2$ for instance can induce bronchoconstriction in aspirin sensitive asthma, and to a smaller degree it may cause bronchodilation, while PGF$_2$$\alpha$ induces bronchoconstriction in asthmatic individuals (Kharitonov et al, 1998).

3.1.2 Effect of cyclooxygenase inhibitors on prostaglandins systems

Several studies have confirmed that inhibition of prostanoid synthesis in vivo and the release of PGE$_2$$\alpha$ from albumin accounts for the therapeutic effectiveness of the aspirin and non-steroidal anti-inflammatory drugs in inflammatory processes (Williams et al, 1991). Since the original observations of Vane, 1971 that aspirin inhibited prostaglandin synthesis, a large number of compounds have been shown to inhibit fatty acid cyclo-oxygenase (Figure 3.1). The efficiency of these drugs, as inhibitors of prostaglandin synthesis, varies between different organs. Aspirin (acetylsalicylic acid) inhibits the enzyme cyclo-oxygenase and thereby irreversibly inhibits the synthesis of prostaglandin, however, the effect on prostacyclin synthesis is more short-lived than that on thromboxane A2 synthesis (Pallister 1998).

The non-steroidal anti-inflammatory drugs, such as indomethacin, naproxen and meclofenamic reversibly inhibit cyclo-oxygenase (Masferrer et al, 1994). Ruffin and his colleague’s studies showed that the low and regular intake of aspirin (acetylsalicylic acid) or non-steroidal anti-inflammatory drugs could affect colorectal cancer by preventing the production of PGE$_2$ and PGF$_2$$\alpha$, which can be found in high levels in colon cancer tissue (Kharitonov et al, 1998).
Figure 3.1: Schematic diagram of the prostaglandins production.

The two pathways of cyclooxygenase and 5-lipoxygenase and the production of their related mediators, prostanoids and leukotrienes, respectively. (Taken from Pathologic Basis of disease, Cotran et al, 1999).
The detection of prostaglandins in serum does not necessarily mean it is an active secretion from local cells due to stimulation. Determining of serum bound PGE$_2$ released by aspirin is unsuitable for diagnosing aspirin sensitive asthma, because it showed non-significant difference between aspirin sensitive and aspirin tolerant individuals (Williams et al, 1991).

### 3.1.3 Prostaglandin F$_2$alpha (PGF$_2$α)

The subscript refers to the number of double bonds; thus PGF$_2$α has two double bonds. The Greek letter “α” refers to the hydroxyl group orientation (Figure 3.2). The main effect of PGF$_2$α is the contraction of smooth muscles. This is due to its action on FP-receptors which when released in corpors luteum and smooth muscle, also causes bronchoconstriction, and luteolysis.

Several studies have shown that PGF$_2$α generally seems to enhance the effects on target organs following adrenergic stimulation.

![Figure 3.2: The chemical structure of PGF$_2$α.](image-url)
PGE₂ has an important anti-inflammatory and brochoprotective role in airways. It inhibits the release of chemoattractants (LTB₄), and inhibits the mast cell mediators in vitro (Celik et al, 2001). In normal subjects it may causes bronchodilation (Kowalski et al, 2000), yet clinical evaluation of this marker does not clarify the mechanisms involved (Williams et al, 1989, Celik et al, 2001).

Williams and his co-worker in 1989 and 1991 measured plasma PGF₂α level using radioimmunoassays. These studies showed that the plasma PGF₂α level was significantly higher in aspirin sensitive patients after the administration of aspirin, in contrast; aspirin tolerant asthmatics had lower levels of PGF₂α (Kurosawa et al, 1990).

A further study, carried out by Kurosawa M, 1990, measured the level of PGF₂α from bronchial atopic asthmatic patients, he found significantly higher levels of plasma PGF₂α in atopic asthmatic patients compared with non-atopic individuals.

It was reported that aspirin seems to release a bronchoconstricting prostaglandin in plasma, and when examined in vitro it was greater in aspirin induced asthma as compared with healthy volunteers. Other studies examined PGF₂α levels in urine. Both of them found the amount of PGF₂α were high in atopic patients in general (Morrow et al, 1990, Dworski et al, 1999).
3.1.4 Drug and serum protein interactions

Many drugs exist in plasma mainly in bound form with plasma protein at therapeutic concentrations. The most important plasma protein in relation to drug binding is albumin; this binds many acidic drugs and a smaller number of basic drugs.

Plasma albumin is a predominantly carbohydrate free protein that constitutes more than 50% of total serum protein. Serum albumin has a high affinity for free fatty acid and other anions. It acts very effectively and therefore, it serves as a carrier protein. Several studies have shown that PGs added to human plasma in vitro were found to bind to plasma albumin. Williams 1991 found that albumin is the source of PGF₂α released from plasma by aspirin. Plasma prostaglandins are protein-bound and binding decreases with the increasing polarity of the molecule. Drugs and free fatty acid compete for ligand binding sites on the albumin. For instance salicylic acid has been shown to be effective at displacing PGA₁ in rat plasma. Some drug-induced asthma with a high protein binding capacity does not displace PGF₂α from plasma (Williams et al, 1991).

Aspirin (acetylsalicylic acid) is metabolically converted to salicylic acid by carboxylesterases. Salicylic acid is extensively bound to plasma albumin and induces the release of PGF₂α from albumin bound (Miners 1989).
3.1.5 Aims

- To investigate, whether, PGF$_2\alpha$ displacement by aspirin occurs in both atopic and non-atopic aspirin sensitive subjects.

- To determine if PGF$_2\alpha$ could be used as a marker for aspirin and salicylate sensitivity.
3.2 Methods

3.2.1 Patients

Forty-two subjects were examined in this study, 18 males and 24 females were divided into 4 groups, 16 atopic aspirin sensitive, 9 non-atopic aspirin sensitive, and 6 patients who were atopic but non-aspirin sensitive and 11 healthy volunteers. The age range was 25–60 years.

3.2.2 Blood samples

To analyse PGF$_2$α, the blood was collected using a serum separator tube (SST), and all blood samples were left at room temperature for 30 minutes to clot, then centrifuged for 15 minutes at approximately 1000 rpm. The serum was then removed for analysis, or stored immediately at $-70^\circ$ C until use.

3.2.3 Reagents

The PGF$_2$α Immunoassay was a commercially available kit (R&D systems, Europe Ltd), for quantitative determination of prostaglandin F$_2$α. All the reagents and samples were brought to room temperature before use. The assay was carried out according to the manufacturers instructions.

Lysine-acetylsalicylic acid (Aspisol, Bayer, Newbury, UK) was used in 1.8mg/ml concentration, and PBS was used as the negative control.

3.2.4 Principle of the assay

This assay employs the competitive binding technique in which PGF$_2$α present in samples competes with a fixed amount of alkaline phosphatase-labelled PGF$_2$α.
A polyclonal antibody specific for PGF$_2$α was pre-coated into a micro-plate. Standards and samples were pipetted into the wells and bound to the antibodies. Wells were washed three times, to remove any unbound substances. An enzyme linked polyclonal antibody specific for PGF$_2$α was added to the wells. An enhanced substrate solution was added to the wells to determine the bound enzyme activity. The colour development was stopped and the intensity of the colour measured at 450 nm.

3.2.5 Sample preparation

Serum from each tested individual was separated into two aliquots (150 µl). The first aliquot was challenged with 20 µl of L-acetyl salicylic acid (aspirin), while the second (used as negative control) was treated with 20 µl phosphate buffer saline (PBS). Both samples were incubated at 37°C for 45 min. The samples were diluted 10-fold with assay buffer ED1. PGF$_2$α was subsequently measured as described above. The plate was read using Micro-plate reader (2000 Anthos), using at 450 nm as the detection wavelength and 620 nm as the reference wavelength.

3.2.6 Calculation of the results

The average of the duplicate readings for each standard, samples and subtract was yielded to obtain the optical density. The corrected optical density (OD) and %B/Bo for each standard and sample was obtained from these equations:

- Corrected OD = sample OD – Non-Specific binding (NSB) OD
- % B/Bo = Corrected OD/ corrected Bo OD X 100.
Having obtained the results for all samples, the concentration of PGF$_2$α was calculated by constructing a standard curve and plotting the mean absorbance for each standard on linear y-axis against the concentration on a logarithmic x-axis and a line curve was drawn through the point on the graph. See appendix (1)

The sample result was multiplied by the dilution factor to achieve the neat concentration of the PGF$_2$α before the sample was diluted.

3.2.7 Statistical analysis

The experiment was performed several times using different parameters, to determine the effect of length of incubation time. Statistical analysis data was entered into a Microsoft Excel spreadsheet and imported into Minitab-12. The assay was performed in duplicate for each sample. Data for each treatment group was expressed as a mean ± SE. Differences in mean PGF2α were analysed by the student’s t-test.
3.3 Results

3.3.1 Measurement of serum PGF$_2\alpha$ from examined groups

One of the main aims was to verify whether PGF$_2\alpha$ release from serum proteins could be used as a reliable marker for aspirin sensitive patients. The response to the administration of L-ASA in tested samples is shown in (Figure 3.3).

Aspirin sensitive patients were divided into atopic and non-atopic subjects based on skin test findings, and family histories.

Atopic aspirin sensitive:

There was a difference in the level of PGF$_2\alpha$ detected in the treated serum with L-ASA compared with untreated serum. None of the differences in any of the samples, regardless of age reached statistical significance. However, the subjects in the atopic aspirin sensitive group showed high activity and produced more plasma PGF$_2\alpha$ level pg/ml compared with the 3 other groups.

It was worth noting that the level of PGF$_2\alpha$ was significantly higher in younger patients among female and male, although the interpretation of this analysis maybe limited, due to the small number of tested males in this group.

Non-atopic aspirin sensitive:

All nine patients showed higher levels of PGF$_2\alpha$ in their serum after treatment of the serum with L-acetylsalicylic acid for 45min. The difference in plasma PGF$_2\alpha$ levels was not statistically significant between treated and untreated serum. Again, younger patients (age< 30) have a higher PGF$_2\alpha$
Figure 3.3: Production of PGF$_2\alpha$ in treated and untreated serum with L-ASA in four tested groups.

A.AS: atopic aspirin sensitive group. nA.As: Non-atopic aspirin sensitive. A.nAS: atopic non-aspirin sensitive. Control: healthy volunteers. PBS (phosphate buffer saline) as negative control. L-ASA: Lysine acetyle salicylic acid. Values represent the means ±SE show no significant difference between the 4 studied groups.
level. 319 pg/ml in comparison to those older subjects (age 45-60), the level ranged from (94.2 - 249 pg/ml).

Control group:
There was no statistical significant different between treated and untreated serum (Figure 3.3), and levels of PGF$_2$$\alpha$ were similar to those seen in the patient groups. Sixty five percent of the tested subjects with a high reading were from females. In addition the majority of these were aged over 25 years old.

Atopic non-aspirin sensitive:
There was no significant difference in the production of PGF$_2$$\alpha$ between the treated and untreated serum with L-acetylsalicylic acid in this group. The released PGF$_2$$\alpha$ was also noticeably lower compared with the other 3 groups, but this did not reach significance. Males and females gave similar results in this group (Figure 3.4).

3.3.2 Effect off L-ASA in the release of PGF$_2$$\alpha$ over time
To determine the release of the PGF$_2$$\alpha$ over time, serum was measured after 1, 2, 3 and 4 hours in order to determine whether prolonged incubation with L-acetylsalicylic acid had different effects in the PGF$_2$$\alpha$ release. Four healthy volunteers and 7 atopic aspirin sensitive were tested (Figure 3.4), as well as four non-atopic aspirin sensitive (data not shown).
Figure 3.4: The level of PGF$_2\alpha$ released in serum from healthy subjects, at different time intervals.

The figure shows no significant different after treated the serum with L-ASA, and PBS (negative control) at different times. Except after 4- hours of incubation, it shows there is a significant different between the treated serum with L-ASA and PBS $p=0.005$. The PGF$_2\alpha$ level was higher in serum treated with PBS compared with the same serum treated with L-ASA. Values represent the mean SE ±.
The effects of time on the amount of plasma PGF$_2\alpha$ level produced was run over a similar time period for the three tested groups, shown in (Figure 3.4), and (Figure 3.5).

Atopic aspirin sensitive group

Within this group the incubation of sera with L-ASA for 45 min showed no significant difference between PGF$_2\alpha$ levels when compared to untreated sera. The increase of the PGF$_2\alpha$ was highly observed with the elongation of incubation time, in both treated and untreated sera with L-ASA. The amount of PGF$_2\alpha$ noticeably decreased after 4 hours of incubation with L-ASA. The difference was significant at this time $P=0.005$, and PGF$_2\alpha$ level was decreased on both treated and untreated sera. As shown in (Figure 3.5), the level of plasma PGF$_2\alpha$ released in aspirin sensitive subjects was much higher as compared with the amount of PGF$_2\alpha$ released in healthy volunteers, at similar time periods.

Control group

There was a slight difference between the treated sera with L-ASA and sera treated with PBS. The plasma PGF$_2\alpha$ level was higher in the sera treated with L-ASA as at two time intervals (2hr-3hr). However, this difference was not statistically significant. The PGF$_2\alpha$ levels gradually decreased in the control group with the increase in incubation time when treated with L-ASA. This decrease was greatest after 4 hours of incubation (figure 3.4).
Figure 3.5: Effects L-ASA in the amount of serum PGF$_{2\alpha}$ level released in atopic aspirin sensitive patients

The figure shows PGF$_{2\alpha}$ level released in serum after the incubation with L-ASA at different times. After 4 hours of incubation the difference was significant between serums treated with PBS compared with serum treated with L-ASA, $P=0.006$. 

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Aspirin irreversibly acetylates serine residue of COX enzymes blocking their activity, as a consequence, production of prostanoids (PG, Tx) from arachidonic acid and the physiological functions that prostanoids perform are impaired.

Generally, the half-life of PGs in the circulation is very short, less than 1 minute. Thus, this decreases the chance of detecting PGs in plasma. (Kurosawa et al, 1990).

These compounds are metabolised rapidly, and the primary PGs may not be the most prominent products existing in the plasma through PG pathway. This makes the detection of small quantities of PGs present in plasma difficult due to the half-lifes are generally very short and many methods used are insensitive and non-specific (Kurosawa et al, 1990).

The goal of this study was to investigate previous studies (Williams, et al, 1991, Bennett 2000) that showed the displacement of PGF$_2$α by aspirin from serum protein (albumin). Above all, to check whether PGF$_2$α released in these aspirin sensitive patients could serve as a marker for identifying this group (aspirin induced asthma).

According to the results of these study the PGF$_2$α levels in all samples were generally higher in all the samples compared with those found in previous published works (Williams et al, 1991, Williams et al, 1990, Szczeklik et al, 2001). This discordance could be due to a difference in the methods used to detect PGF$_2$α. In this study ELISA was used, while previous studies used RIA. Secondly, it may relate to storage conditions used in this work. The samples were stored for a long time (more than three weeks) at -70°C. This was done to combine all the patients' samples and run them together.
Among the atopic aspirin sensitive (AAS) and non-atopic aspirin sensitive (nASA) patients the expression of PGF$_2\alpha$ was higher in the treated serum as compared with the untreated serum, in both groups. Unfortunately the difference was not statistically significant. No such significance was found in the other two groups namely atopic non-aspirin sensitive (AnAS) and the control (healthy volunteers).

This outcome confirms the competition for albumin binding between aspirin and prostaglandin. The greater the sensitivity to aspirin, the higher the displacement of PGF$_2\alpha$ from albumin, and consequently, the higher free unbound PGF$_2\alpha$ present in the serum. As the first two groups were aspirin sensitive, the high PGF$_2\alpha$ levels were expected, although this increase was not significant.

Release of PGF$_2\alpha$ seems to be age dependent. Younger tested subjects showed higher amount of plasma PGF$_2\alpha$ level as compared with other tested subjects aged of 40-60, in the four studied groups. An unexpected observation was that the released of serum PGF$_2\alpha$ in the control group was similar compared with the other 3 groups. This group was expected to have lower levels. However, it is possible that although the control group comprised of healthy volunteers, it cannot be excluded that they may have other conditions that may have contributed to the PGF$_2\alpha$ expression in their sera.

No significant difference in the amount of plasma PGF$_2\alpha$ level was detected among the four studied groups after the addition of aspirin. This implies that the measuring of plasma PGF$_2\alpha$ levels before or after aspirin incubation in aspirin sensitive patients is not a suitable test to predict or detect this group.
This data confirms the findings of previous study (Bennett 2000), that aspirin has no effect on levels of PGD$_2$ and PGF$_2\alpha$, but conflicts with other reports that showed the concentration of plasma PGF$_2\alpha$ was greater in aspirin sensitive patients compared with aspirin tolerant ones (Williams et al, 1991, Plaza et al, 1995).

Despite the fact that the data gives no clear indication for the role of PGF$_2\alpha$ in aspirin induced asthma *in vitro* it is unclear whether this reflects what is actually happening *in vivo*. It is possible that local concentration of PGF$_2\alpha$ in tissues may be more readily displaced from protein by aspirin.

In conclusion, treatment of serum with L-ASA at various incubation times had no significant effect on the amount of PGF$_2\alpha$ released. The results of this work highlight that the release of serum protein PGF$_2\alpha$ is likely not to be of useful value in predicting subjects who are aspirin sensitive.
Chapter Four

Assessment of IL-4 expression as a marker for the diagnosis of AIA and salicylate sensitivity.
4.1 Overview

Aspirin induce asthma (AIA) and salicylate sensitivity have a number of similarities with type I hypersensitivity. IL-4 is one of the cytokines causing immunoglobulin E (IgE) switching in B cells, this leads to inappropriate IgE synthesis, which characterizes atopy (Gemou-Engesaeth et al, 2002). Cytokines are produced from several different cells and named according to the source from where are they produced e.g. monokines are produced by mononuclear phagocytes. Most cytokines in specific immunity are produced by activated T lymphocytes. The secreted cytokines determine the functions of the T cell (Callard 1994).

$T_H^1$ cells induce primarily cellular (type 1) immune response, while $T_H^2$ cells promote humoral (type 2) immune response. $T_H^1$ cells secrete IL-2 and IFN-$\gamma$ and promote delayed type hypersensitivity reaction, macrophage activation and cytotoxic cell responses. These responses direct cellular immune inflammatory reactions and provide primary host immune defences against intracellular pathogens. In contrast, $T_H^2$ cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13 and promote B-lymphocytes responses and antibody synthesis (Minty et al, 1997, Romagnani 2001).

Several studies have demonstrated that basophils, which similarly infiltrate allergic lesions, have the ability to make and secrete cytokines after activation with either non-specific stimuli or specific antigen, particularly IL-4 along with that eosinophils and lymphocytes. Several studies showed that basophils generate high levels of IL-4 protein (Schroeder et al, 1998, Devouassoux et al, 1999, Ying et al, 1994).
4.1.1 General properties of cytokines

Cytokines are small soluble proteins (c.8-80 KDa molecular weight) or glycoproteins, produced by mononuclear cells of the immune system (usually lymphocytes and monocytes), as well as non immune cells that have regulatory functions such as activation, proliferation and differentiation, and cell-cell communication (Leutenegger et al, 1999).

Cytokines have a wide variety of names. This is not surprising, as they have been discovered in disciplines ranging from immunology, virology, and haematology to cell biology and oncology (Table 4.1).

Although cytokines are diverse group of proteins, these molecules share a number of properties:

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviation</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukins</td>
<td>IL</td>
<td>IL-1, IL-2, IL-3, IL4, etc</td>
</tr>
<tr>
<td>Interferons</td>
<td>IFN</td>
<td>IFNα, IFNβ, IFNγ</td>
</tr>
<tr>
<td>Tumour necrosis factor</td>
<td>TNF</td>
<td>TNFα, TNFβ</td>
</tr>
<tr>
<td>Growth factors</td>
<td>GF</td>
<td>NGF, EGF</td>
</tr>
<tr>
<td>Colony stimulating factors</td>
<td>CSF</td>
<td>M-CSF, G-CSF, GM-CSF</td>
</tr>
<tr>
<td>Chemokines</td>
<td>-</td>
<td>IL-8, RANTES, MCP-1, MIP1</td>
</tr>
</tbody>
</table>
Cytokines like other polypeptide hormones exhibit different effects on various target cells. They initiate their action by binding to specific receptors on the surface of target cells. The relative target cell may be the same cell that secretes the cytokine (autocrine action), a near by cell (paracrine action), or a distant cell that is stimulated via cytokines that have been secreted into the circulation (endocrine action). Although the contributory cytokines, in processes such as haematopoiesis, wound repair and inflammation seem to have been largely identified, information on the operation of the cytokine network in simple experimental systems is still far from complete. (Callard 1994).

The cytokines represent suitable models for studying the mechanisms regulating gene expression, since the expression of cytokines appears to be tightly regulated by restricted types of cells and inducers such as IL-4 is secreted by lymphocyte (Th2) cell and basophil (Roitt et al, 1998).

4.1.2 Importance of Cytokine studies

Cytokines are the major orchestrators of our host defence processes, and as such, are involved in response to exogenous and endo-genous (insults), repair and restoration of homeostasis. The study of cytokines elucidates the mechanisms underlying pathophysiological processes. Cytokines mediate host responses to invading organisms, tumours, and trauma (Arai et al, 1990). Therefore detection and characterisation of cytokines in disease states may provide useful diagnostic tools.
4.1.3 Role of Interleukin – 4 (IL-4) in allergic disease

Among many cytokines produced by \(T_H^2\) cells, mast cells and Basophils, IL-4 has emerged as important effector molecule; it expresses as a 15-20-kDa glycoprotein and exists as a dimer (Feghali et al, 1997, Bacharier et al, 1998). IL-4 has been known as the “prototypic immunoregulatory cytokine”, like many cytokines, it can affect several target cells in multiple ways. Its has a main role in regulating the antibody production of IgE by activation of B cells and the development of effector T-cell responses e.g. differentiation of naive CD4 T cells into \(T_H^2\) population rather than into \(T_H^1\) population. In addition, IL-4 increases surface CD23 (FRIII), MHC class II and IL-4R expression on B cells and monocytes, as well as increasing VCAM-1 expression on endothelial cells (Kay et al, 1995, Humbert et al, 1997).

Many studies have revealed that IL-4 is essential for allergic disease and critical determinant of the outcome of infection or autoimmunity in certain contexts (Kay et al, 1995). Elevated IL-4 concentrations have been detected in the serum of asthmatic patients. These reports show that the stimulated blood cells from atopic children with asthma produce more IL-4 than non-atopic control (Roponen et al, 2001). IL-4 has an important role as it down regulates \(T_H^1\)- mediated inflammatory responses and it may have immunosuppressive or even preventive potential in autoimmune disease (Roponen et al, 2001). However, the difficulties in detecting antigen stimulated or spontaneous IL-4 secretion are related to in vitro consumption of IL-4, because of the wide distribution of IL-4 receptors on different cell types. In addition, IL-4 is secreted in low, but biologically significant amounts. Even after polyclonal stimulation IL-4 concentration is very low (1000-fold lower than IFN\(\gamma\) concentration) (Ekerfelt et al, 2002).
4.1.4 Salicylates and IL-4

Upon ingestion of aspirin or NSAIDs, cysteinyl leukotrienes are released and this leads to worsening of the asthmatic symptoms (Sousa et al, 1997). Several studies, showed that salicylic acid which is the active ingredient in aspirin suppress a number of enzymatic activities, and signalling through IL-4R (receptor), which participates in the development of allergic responses (Perez-G et al, 2002). One proposal is that aspirin interferes with arachidonic acid metabolism by inhibiting COX-1 and COX-2 pathways and/or increasing the lipoxygenase release of leukotrienes (Varga et al, 1999). A previous study has shown that allergic patients with seasonal rhinitis demonstrate an increase in local expression of \( T_H^2 \) type IL-4 and IL-5 but not \( T_H^1 \) type IL-2 and INF-\( \gamma \) cytokines (Varga et al, 1999).

4.1.5 Cytokine Analysis

Cytokines can be analysed either at the protein level using Bioassays, Enzyme Linked Immunosorbent Assay (ELISA) and Immunohistochemistry or at mRNA level using RNAsse protection assay, Northern blotting or Reverse Transcription–Polymerase Chain Reaction (RT-PCR). Choosing the method for analysis of mRNA depends on the amount of the RNA available and on the level of gene expression (Dollman et al, 1991). For the purpose of this study RT-PCR was used to determine cytokine IL-4 gene expression. This technique was chosen as it only required a small sample of blood (<1.0 ml) to be taken from the patient and the technique could be prepared in a relatively short time (2-3 hrs). Since some disease states can be attributable to defects in the transcriptional process, quantifying mRNA can provide insights into the aspects contributing to it.
4.1.6 Aims

- To determine if AIA individuals secreted more IL-4 in response to aspirin and NSAIDs when compared to healthy controls.

- To determine if changes in IL-4 levels could be attributed to differential IL-4 gene expression.
4.2 Methods & Materials

4.2.1 Collection and storage of blood samples

Sodium-heparinized venous blood was obtained from healthy volunteers, aspirin sensitive patients and subjects with a history of grass pollen allergy. All the subjects were skin prick tested to determine atopic status. Peripheral blood mononuclear cells (PBMC) from heparinized venous blood were isolated by density gradient centrifugation on Ficoll-Hypaque. For RT-PCR experiments, the blood was placed into labelled eppendorf. To some added L-acetyl salicylic acid (L-ASA) and incubated for 30 min at 37°C before freezing and stored at −80°C, other incubated with PBS reagents and stored at −80°C.

4.2.2 Isolation of PBMC for lymphocyte culture

Whole blood (7ml) was carefully laid onto 7ml of Histopaque-1007 (Sigma Diagnostics®). Centrifuge at 450xg for 30min at room temperature. After centrifugation the upper layer (plasma) was discard. Mononuclear cell layers were aspirated with a Pasteur pipette, and transferred into a clean sterile universal tube. PBS (10ml) was added and mixed by gentle aspiration. The leukocyte was centrifuged at 400xg for 10min. The supernatant was aspirated and discarded. The cell pellet was reused with 10ml of PBS and aspirated with a Pasteur pipette. After centrifugation at 400xg for 10min the cells pellet was resuspended with 0.5ml of PBS. Leukocytes were counted using a coulter® MicroDiff apparatus. Isolated leukocytes were counted in order to assess efficacy of isolation protocol and to optimally adjust leukocyte concentration (5 x 10⁶cells/ml) before incubation.
4.2.3 Culture conditions

Lymphocytes were washed with PBS twice, then the cells were cultured at cell densities of $5 \times 10^6$ cells/ml and the number of cells was $100 \times 10^3$ per well. These cells were incubated in RPMI medium supplemented with 50U/ml of penicillin and 50µg/ml of streptomycin (Curiel et al, 1999). In addition, different concentrations of phytohemagglutinin (PHA) 5µg/ml, 10µg/ml, and 100µg/ml as an activator, and grass pollen 1.25µg/ml, 2.5µg/ml and 5µg/ml were added. Control cultures received medium alone. Cells suspension was added to give a final volume of 200µl. Calcium-ionophore (1mg/ml; A23187, sigma) (Laan et al, 1998. Cianferoni et al, 2000) was added as a stimulator for IL-4. This was not used currently. The cells were incubated in sterile 96 round bottom micro well culture plates (Cell Wells, Coming Glass Works, Coming, New York 14831) at 37 °C in humidified incubator with 5 % CO$_2$ for 3 days. Cell free supernatants were then removed and stored at -80°C. The cells were washed 3 times, after centrifuging the cells and stored it if not use at 80 °C.

4.2.4 Measurement of IL-4 by ELISA

The level of IL-4 in the culture supernatants was determined by ELISA kits (Quantikine® HS, R&D Systems Europe Ltd) according to the manufacturer’s instructions. The assay sensitivity and the lower detection limit for IL-4 were 0.25pg/ml.
4.2.5 mRNA analysis Techniques

4.2.5.1 RNA extraction

The extraction carried out using the Ambion RNAqueous “Phenol Free Total RNA Isolation Kit” and the Ambion RNAqueous “Blood Module”. The method was carried out according to the manufactures instructions; other solutions are described in Appendix-1. Briefly:

A 1.2ml of RBC Lysis solution was placed in 2ml RNase-free polypropylene tube and 400µl of anticoagulated whole blood was added to the tube. The mixture was left on ice for 10 minutes; the solution was vortexed briefly 3 times at regular interval. The mixture was centrifuged at 13,000 rpm for 30 sec this allowed the formation of leukocyte pellets. The supernatant fluid was removed by pouring it off. 1ml of RBC lysis solution was further added to the pellet and followed by vigorous vortex. Centrifugation again saw the isolation of the leukocytes at the bottom of the tube.

By using a pipette, lysed RBCs were removed and no more than 30µl of the residual fluid was left behind. 350 µl of Lysis / Binding Solution was added to the pellet and the tube was vortexed vigorously. At this stage the colour of the lysate ranged from pale to dark amber. A 350 µl of 64% ethanol was added to the lysate and it was mixed gently followed by centrifugation for 3sec. The mixture was pipetted on to the filter cartridge into an RNase free collection tube supplied, and centrifuged at 13,000 rpm for 1min, to allow the lysate/ethanol mixture to pass through to the filter.

Wash solution 1 (700 µl) was added to the filter. Followed by 15sec centrifugation at 13,000 .500 µl of the wash solution 2/3 was applied and the same centrifugation was performed. The filter cartridge was applied to a fresh collecting tube. 30 µl of Elution solution was added to bind to the filter. The tube and the filter apparatus was
incubated in a heat block set to 65°C for 10 minutes, then followed by centrifugation at 13,000 rpm for 1 minute. The elution stage was repeated twice using the same collection and Elution tube leaving 60 μl of RNA rich suspension, which was stored at −70°C to limit the action of any endogenous Rnase, or was immediately used for the next step.

4.2.5.2 DNAase treatment of RNA

For all techniques used in this study, RNA was DNAase treated in order to completely remove any traces of DNA contamination. DNA contamination may be a major source of false positive or negative findings in gene expression analysis. This removal of contamination is essential to insure that mRNA and not genomic cDNA products are analysed. A DNA Free kit (Ambion) was used, and the procedure was carried out according to the manufactures instructions.

4.2.5.3 Purity of RNA

The purity and integrity of isolated RNA is critical for the effective use in RBA analysis techniques like, the RT-PCR, Northern blotting, etc. As most of the work done in this study focused on the expression of mRNA it was imperative that RNA be intact and quantified correctly. For that, two techniques were routinely used, spectrophotometric analysis and denaturing agarose gel electrophoresis.

20 μl of RNA was added to 480μl of H2O (1:25 dilution) and analyzed in a (Cecil 1021) scanning spectrophotometer against H2O blank. The optical density (OD) reading at 260nm (A260) and 280nm (A280) were noted and the appropriate calculations were made to compensate for the dilution factor and conversion factor of nucleic acid in RNA format. The formula for RNA quantification is as follows:
RNA μl/ml concentration = A 260x 40 (conversion factor for RNA) x 25 μl (dilution factor). Samples with OD readings of less than 0.05 were not processed (due to low yield). The ratio of (A260/ A280) should always be between 1.8 - 2.0 for pure nucleic acid samples.

4.2.5.4 Denaturing agarose gel electrophoresis

As RNA has a high degree of secondary structure, it is important to use denaturing gel rather than a normal agarose gel. By adding formaldehyde, the secondary structure is disrupted and RNA molecules can be separated by charge migration according to their size. Two bands ribosomal (S28 – S18) should appear on the stained gel.

The gel was made by dissolving 0.2g of agarose (RNA Agrose Fisher Scientific) in 17mls of Rnase free water. To that was added 2mls of 10x MOPS buffer and 1ml of 37% formaldehyde. After adding 3μl of 1mg/ml ethidium bromide, the gel was poured into a mini gel tray. After solidification, the gel was submerged in 1x MOPS buffer ready to run. Samples were prepared: 5μl of RNA was mixed with 5μl of RNA loading buffer solution (RNA Queous Ambion®) to a final volume of 10μl. The mixture was incubated for 5 minutes in 65°C water bath to denature RNA. Samples were loaded into the gel and 80 volts for 1hour. The gel was placed on top of UV transilluminator and photographed by (Gel DOC 2000, Bio- RAD) & (Mitsubishi pgl Camera).

4.2.6 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The advantage of the PCR lies in its ability to amplify small amounts of DNA exponentially. It is involves: a) Denaturation of DNA into two separate strands, b) annealing of flanking oligonucleotide primers on the separated strands, c) and extension of the primers using thermostable polymerase enzyme (Figure 4.1). These
three steps (denaturation, primer binding and DNA synthesis) represent a single PCR cycle, and each step is carried out at a discrete temperature (e.g. 94°C to 98°C, 37°C to 65°C and 72°C.

4.2.6.1 Reverse transcription (RT)

After extracting the RNA from the whole blood, it was immediately converted to cDNA by reverse transcriptase, to make it more stable and so it was possible to amplify it. The cDNA was prepared using the following protocol. Out of the 60μl purification RNA products, 0.150ng (5 μl) μl was placed into an eppendorf tube together with 0.5μl of random hexamers and 13.75μl of RNase free water. A primary heating stage, to 70°C, for 5 min, and cooled immediately. To the above sample 2.5 μl of 5x RT buffer was added along with 1.25μl of dNTPs, 1.0 μl of reverse M-MCV. RNase inhibitor (1.0μl). Elimination of the RT from an additional tube, containing all other components of the mixture, constituted a negative control. The samples were incubated at room temperature for 10 min, then 42°C for 1 hour and at 70°C for 15 min. The samples were then tested for the presence of cDNA using a housekeeping gene (β-actin) in Homo sapiens actin, and constitutes expressed in all cells.
Figure 4.1: The principle of PCR reaction and cDNA amplification
(Taken from Passarge 1995)

A. Polymerase chain reaction (PCR)

B. cDNA amplification

Complementary DNA single strands as template for DNA synthesis

Amino acid sequence (partial) of a protein

1. Different RNAs

2. Reverse transcriptase + oligo (dT)

3. Alkaline hydrolysis

4. Sense oligonucleotide as primer

5. Anti-sense oligonucleotide as primer

6. New DNA

Further specific amplification of new DNA
4.2.6.2 Polymerase Chain Reaction (PCR)

Once cDNA was made in the RNA samples, it was then used for subsequent PCR detection. PCR amplification was performed by adding 5 µl of each cDNA sample to a final reaction mixture of 25µl containing:

- 2.5 µl of 10x Mg free buffer
- 1.5 µl of MgCl₂
- 1.25 µl of dNTPs
- 8.75 µl of RNase free water
- 1 µl of Taq polymerase
- 2.5 µl of each primer (sense and anti sense of IL-4) Table 4.2.

A blank control (water), no (cDNA) was added. Tubes were spun briefly, overlaid with a drop of oil and transferred to a Cetus thermal cycle (Table 4.3).

Table 4.2: The sequences of different types of investigated primers IL-4 and β-actin.

<table>
<thead>
<tr>
<th>mRNA species</th>
<th>Size (bp)</th>
<th>Primers</th>
<th>Primers sequence (5'-3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Antisense</td>
<td>5'-GTTGTGAGCCTGGACCTCATTAC-3'</td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>317</td>
<td>Sense</td>
<td>5'-CTCCCCCTCCTGTTCTCCTC-3'</td>
<td>Naohiro et al, 1992</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense</td>
<td>5'-TTCCTGCTCGAGCGTTCATC-3'</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>314</td>
<td>Sense</td>
<td>5'-CTCCTTAATGTTCAGGCTATCTTC-3'</td>
<td>Humbert et al, 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense</td>
<td>5'-GGGGCGCCGAGGACCA-3'</td>
<td></td>
</tr>
</tbody>
</table>
4.2.6.3 Analysis of PCR products

The most common method of analysis for the product of PCR is via gel electrophoresis. It is a semi-quantitative analysis. A 2% agarose PCR gel was produced by adding 0.3g of agarose to 3.0ml of 10x MOPS and 27ml of RNase free water. A final concentration of 2.0µl of ethidium bromide (Sigma, Ltd, UK) was added. The gel was then poured and left for 20-30min to set. An 8 well comb was inserted for the PCR gels, from each sample 5 µl of DNA product and 5 µl loading dye were mixed and placed straight into the wells. Electrophoresis at 80V for 30-45 min terminated the preparation stage.

The gel was viewed using a transilluminator. Subsequent gel photography and densitometry allowed replication of the gel and semi-quantification of the bands respectively (Gel DOC 2000, Bio- RAD) and (Mitsubishi pgI Camera).

4.2.7 Cells suspension (for RNA enrichment).

The high number of red blood cells present in whole blood makes it a difficult substrate to work with when isolating mRNA, although mRNA isolation from the
whole blood is possible without removing the red blood cells. It was decided to separate the leukocytes from the whole blood to get more specific binding and reduce the contamination factor from unwanted cells. An alternative procedure is to separate the desired leukocyte population by using “Ficoll-pique” centrifugation; the method was performed as described previously in section (2.2.5-d) (Miroli et al, 1986).

4.2.8 Measuring IL-4 level released by different agents

The previous results reconfirmed the true observation seen in IL-4 gene expression was upregulated in response to a one hour exposure to antigen (D.pteronyssinus). These stimulation periods were optimal for studying cytokine mRNA expression. Although, IL-4 has been long associated with early “immediate” response especially to not sure. On the other hand, not much is known about how IL-4 works especially concerning its expression in response to aspirin. The following time course studies were therefore designed. PMNs cells were activated by time intervals of 1h, 3h, 6h, 16h, and the treated as well as untreated cells were processed for RNA isolation and RT-PCR, as described in materials and methods.

4.2.9 Statistical analyses

Statistical evaluations were performed using the nonparametric method, and Mann-whitney U-test was performed where applicable. Minitab software (Minitab inc) was used. Data are expressed as the mean Standard Deviation (SD).
4.3 Results

4.3.1 Release of IL-4 after stimulation with grass pollen

In order to determine the release of IL-4 protein from lymphocytes activated by specific allergen grass pollen out side of the pollen season on atopic volunteers with proven sensitivity. In order to evaluate the efficiency of lymphocytes culture and antigen stimulation as a means of inducing IL-4 production, 6 subjects with known sensitivity to grass pollen, 6 atopic controls with no grass pollen sensitivity and 5 non-atopic subjects were included. Phytohemagglutinin (PHA) was used as a positive control and PBS was the negative control. After 3 days of culture, IL-4 protein release was detected from stimulated lymphocyte cells with grass pollen and PHA separately. PHA gave a significant release of IL-4 in all the study groups, compared to the negative control. The release of IL-4 after treatment with PHA was noticeably higher compared with cells treated with grass pollen. (Figure 4.2B)

Grass pollen gave significantly higher levels of IL-4 release in the grass pollen sensitive subjects, compared to the other group. The difference was statistically significant between subjects who were grass pollen sensitive and healthy volunteers (control) at all concentrations. This difference was statistically significant (p<0.05). However, the expression of IL-4 on the treated samples with grass pollen was very low compared with the amount of IL-4 protein released after treatment with PHA (figure 4.2B). This was not unexpected since PHA is a T-cell mitogen whereas grass pollen only stimulates antigen specific T-cell clones.

The atopic controls gave increased levels of IL-4 with grass pollen compared to the negative control (non-atopic), however this increased did not reach significant level. This may be due to potential cross reaction with other antigens to which these subjects
may be sensitive. The difference in the amount of IL-4 protein released between the 3 groups was low which is probably due to the small number of individuals analysed in this study.

**Figure 4.2A: The production of IL-4 after activating the cell cultures with different stimulus grass pollen.**

The histogram illustrate the effects of grass pollen on the released of IL-4 protein from treated sera from atopic grass pollen sensitive patients, atopic and non-atopic patients as positive and negative control respectively. Values are expressed as mean ±SD.
Figure 4.2B: Production of IL-4 protein levels following the addition of phytohemagglutinin (PHA) to cell culture in three different groups

The histogram illustrates the effects of PHA on the released of IL-4 protein from atopic grass pollen sensitive, atopic and non-atopic as positive and negative control respectively. Values are expressed as mean ±SD.
4.3.1.1 Effects of acetylsalicylic acid on the IL-4 protein production

Experiments were then performed to examine the effects of L-ASA on the release of (IL-4) from lymphocytes in aspirin intolerant, and atopic non-aspirin sensitive subjects. L-ASA gave no significant increase in IL-4 release in any of the studied groups. There was a slight increase in IL-4 release at 1.8mg/ml in all groups but this was not significant and the levels of IL-4 were significantly lower than those seen with grass pollen p<0.05. In general, none of the concentration of L-ASA used in our study affected the production of IL-4 in the 3 study groups.

**Figure 4.3: Effects of L-ASA on the released IL-4 protein**

The histogram illustrate the effects of L-ASA on the released of IL-4 protein from atopic non-aspirin sensitive, non-atopic aspirin sensitive patients, and healthy volunteers (control). The cell culture (lymphocytes) was treated with different concentrations of L-ASA. Values are expressed as mean ±SD.
4.3.2 RNA extraction and DNAase treatment

The extracted RNA was run on a 1.5% denaturing agarose gel to check its integrity. Due to the low quantity of RNA extracted from patient samples, the RNA lanes were only just visible. To check whether the RNA extraction method and gel electrophoresing technique were functional, RNA was also extracted from Monocytic cell line (MM6) (RNA samples of MM6 was given by Maninder Alhwalia). These cells usually have a higher RNA yield than patients samples (Figure 4.4 A-B). Two faint S28 and S18 ribosomal RNA lanes were seen in 5 patients RNA samples with no degradation (Figure 4.4A). Ribosomal lanes were also seen in MM6 (Figure 4.4 A-B).

The DNA contaminated bands were present in all extracted mRNA (Figure 4.4A). These samples were treated with DNA-free™ (Ambion), to remove contaminated DNA lanes from RNA preparations. Figure 4.4B shows the results of RNA extraction after DNAase treatment. Clear lanes were seen in all samples without the DNA contaminated lanes. After RNA extraction, the samples were used for RT-PCR reaction to detect the presence of IL-4mRNA.
Figure 4.4-A: Total RNA extraction from treated samples with L-ASA.

The 28S and 18S lanes indicate intact undegraded RNA. The DNA contaminated lanes are very obvious in these samples. Samples 1, 2, 4, 5 were isolated from whole blood. Sample 3 is from MM6 cells.

Figure 4.4-B: Total RNA extraction from treated samples with L-ASA.

The DNA contaminated lanes disappeared after the samples were treated with DNA-free™.
4.3.3 Expression of IL-4 mRNA in aspirin sensitive patients

The RNA was extracted from 34 samples (each samples was treated with L-ASA and incubated at different points). After RT-PCR, no cytokine expression was seen, regardless of whether these patients were atopic or non atopic. No PCR product of IL-4 mRNA (352 bp or 317 bp) was detected in any of the patient's samples. Figure (4.5) shows the results of 8 samples. This could either be attributed to the low amount of extracted RNA (0.045µg/ul), or the inability of PCR to amplifying enough products to be visualized on gel.

A different number of cycles were run between 30 to 40 cycles, however none showed the IL-4 mRNA PCR product, again no product was observed, suggesting the PCR technique was not optimal. Because no IL-4 was obtained from RT-PCR runs, amplifying β-actin (house keeping gene) was used to check whether our RNA samples had suffered any degradation before RT-PCR and also to determine the efficiency of the reagents used in the amplification.
Figure 4.5: The PCR amplification of IL-4 RNA isolated from whole blood

Amplification product, primer dimers were visible in samples (1-6). (M) is the molecular weight leader, while (7) represent the negative control (no cDNA added) and no band or primer dimer occur. This indicates no contamination during the experiment.
4.3.4 The expression of β-actin in treat sample with L-ASA

Actin is a protein of the cytoskeletal system (in microfilaments) found in all eukaryotic cell types that allows movement of cells and cellular processes. It works in conjunction or in tandem with other components (myosin) of the system. 15% of its activity is to produce movement of cells (Smith and Wood, 1993). Six kinds of actin are known, four of α-actin is found in muscle cells, and two are β and γ, which are found in non-muscle cells (Smith and Wood, 1993).

β-actin PCR was performed at the same time on all samples using the same reaction mix to ensure similar conditions. β-actin (lane314 bp) was detected in all samples. Figure 4.6 shows the results of β-actin lanes of the samples. There was a variation in the expression patterns; some were more intense than others. The lanes (2, 3, 4, 5) were products of mRNA extracted from fresh samples, while the other lanes (1, 6) were products extracted from frozen samples. This suggests that freezing samples has an adverse effect on the lane product intensity. Lane 7 was the negative control sample; no lane appeared, indicating lack of contamination.

The samples used in this experiment were for patients who were aspirin sensitive. The lanes that represent those patients are 1,3,4 and 6. Lanes 1 and 3 represent atopic aspirin sensitive patients, while lanes 4 and 6 represent the non-atopic aspirin sensitive patients. Lanes 2 and 5 represents the products extracted from fresh samples from healthy volunteers (Figure 4.6).
Figure 4.6: PCR amplification of β-actin (314bp) from RNA extracted from whole blood

Agrose gel electrophoresis showing the results of β-actin (314bp) RT-PCR product extracted from aspirin sensitive (1,3,4,6 lanes) and non-aspirin sensitive patients (2,5 lanes). Lane (7) was a negative control no cDNA was added. M=100bp ladder
β-actin was also carried out on 4 patients (fresh samples) and analysed. Samples were prepared at different incubation times (1,3,6 hours) after addition of aspirin (ASA) and run on the same RT-PCR thermal cycle. This investigated the possibility of alterations that might result from exposing the samples to different incubation times. The PCR products of these samples are represented in Figure 4.7. Each 8 fresh blood sample was incubated at 1,3,6 hours at 37 °C and RNA was extracted. One set of samples was frozen before incubation with aspirin (ASA).

Incubation time did not seem to affect the quality of PCR product obtained. If the blood samples were frozen prior to RNA extraction no PCR product was detected. This suggested that during freezing of the blood the RNA suffered degradation (lanes 7,8,9) Figure 4.7.
Figure 4.7: Effect of incubation time and freezing on β-actin amplification from whole blood

The PCR products of β-actin (314bp). Samples were incubated with aspirin (ASA) for 1,3,6. Groups incubated: lanes 1,4,10 were fresh blood of atopic aspirin sensitive. Lanes 2,5,11 was fresh blood of non-aspirin sensitive. Lanes 3,6,12 was fresh blood of atopic non-aspirin sensitive. Lanes 7,8,9 frozen blood sample from atopic. Lane 13 was negative control for sample (1) no cDNA added.
4.4 Discussion

This study, aimed to investigate the effect of aspirin (L-ASA) on the release and up-regulation of potent anti-inflammatory mediators such as IL-4. In order to pursue this aim IL-4 was measured in whole blood using the techniques of ELISA and RT-PCR respectively.

The data obtained from ELISA showed that when the sera of atopic patients who are grass pollen sensitive were treated with grass pollen for 30 min at 37°C the released of IL-4 detected was higher and statistically significant (p= 0.05) when compared with the amount of IL-4 obtained from control subject (atopic non-grass pollen sensitive and healthy volunteers)(Figure 4.2A).

Phytohemagglutinin (PHA) is a T-cell mitogen (stimulator) used as a positive control and to enhance the release of IL-4 from the cultured lymphocytes. The level of IL-4 protein detected from the samples treated with PHA was higher compared with the samples treated with grass pollen only. From these results, it is possible to conclude that IL-4 plays a role in allergy, however the aim of the study was to see if IL-4 could also play a role in aspirin sensitivity. Therefore, the samples of atopic non-aspirin sensitive and non-atopic aspirin patients were treated with L-ASA for 30 min at 37°C.

To determine IL-4 levels sera were treated with L-ASA (aspirin), which showed no significant increase in the amount of IL-4 detected compared with non-treated sera. Different concentrations of L-ASA were used (1.8mg/ml, 3.6mg/ml, 0.18mg/ml, 0.36mg/ml); none of these concentrations of L-ASA showed an increase in IL-4. This suggested that IL-4 is not induced in patients with AIA.
It was worth also suggested to determine if there was a change in IL-4 gene expression when the samples of aspirin sensitive patients were activated with aspirin (L-ASA), since no significant difference was seen in IL-4 expression using ELISA technique. Several studies used animal (rats), (the primers they used shared 45% with human IL-4 mRNA primer). They have succeeded in yielding IL-4 mRNA. However, one of these studies used multiple stimulators such as phytohemagglutinin (PHA) and Ca-ionophore to induce IL-4 in combination with L-ASA production in whole blood (Noble et al, 1995, El-naggar et al, 1998).

The failer to detect IL-4 and to obtain RT-PCR product cannot explain the lack of expression in the system. However, many possibilities can be suggested; a threshold phenomenon caused by low level of IL-4 mRNA in the tested samples. The amount of mRNA that was extracted from patient samples was not enough to yield RT-PCR product. The mRNA only represents 1-2% of total RNA; therefore out of 0.045 ug/ul of total RNA yielded only 0.9ng/ul mRNA was obtained (Beckler et al, 1996). Also it is possible that incubation times of 1-6 hours with ASA that were applied in these experiments may not have been long enough to activate the expression of this gene; Hence, the lack of RT-PCR products.

Detection of β-actin expression in all the RNA samples indicates that RNA and RT-PCR experimental conditions were working. Both frozen and fresh samples were used it this study. It was noted that the expression of β-actin was stronger in the fresh samples compared to the frozen ones (Figure 4.7 lanes 7-9 shows that the freezing could cause some degree of degradation; this may explain the lower expression in the frozen samples). As it was difficult to detect IL-4 in either the fresh or frozen sample, the extraction of β-actin product from all the samples agrees with the previous
conclusion regarding the low expression level of IL-4, and excludes the possibility of a lack of expression due to degradation.

RT-PCR work is in agreement with the ELISA test. In both tests it was unable to detect arise in IL-4 mRNA or protein from ELISA technique. This indicates that IL-4 is not induced by activated the blood with aspirin in sensitive patients to aspirin and salicylates (Figure 4.3).

In conclusion, this study did not demonstrate any change in the expression of IL-4 in treated samples with L-ASA, which may suggest that IL-4 production may not play a role in this condition. Therefore, IL-4 does not appear to be useful as a marker of aspirin induced asthma (ASA).
Chapter Five

The effects of aspirin in nasal challenge and the measurement of inflammatory
5.1 Overview

Aspirin induced asthma (AIA) is a life threatening condition involving bronchoconstriction, and nasal congestion. It is induced in more than 10%-15% of adult asthmatics by a small amount of aspirin, cyclooxygenase inhibitor or anti-inflammatory drugs (NSAIDs). Half of these patients require maintenance therapy with systemic corticosteroids. A large survey of asthmatic patients requiring ventilation has shown that 25% were found to be aspirin-sensitive; yet diagnosis of these patients is a great challenge (Szczeklik et al, 1998).

Aspirin and NSAIDs currently play a major role in the treatment of coronary artery disease (CAD), asthma and other respiratory diseases. It has been shown that a soluble form of aspirin significantly inhibits the bronchial obstructive response induced by a variety of stimuli, such as histamine, allergens, neurokinin and adenosine. However, many patients are denied treatment with aspirin because of the history of aspirin and NSAIDs-induced urticaria and angioedema. (Szczeklik et al, 1998).

Subjects who suffer from AIA frequently develop chronic rhinosinusitis with nasal polyps (Hosemann 2000, Nizankowska et al, 2000). The first symptoms that appear in the majority of patients who become aspirin-sensitive are related to a flu-like post viral infection. Symptoms are seen during the third or fourth decade of life, and are characterized by discharge from the nose, which is often watery. Two years after the appearance of rhinitis, the first symptoms of asthma appear (Szczeklik et al, 2000). Intolerance to aspirin becomes evident several years later and nasal polyps maybe be diagnosed at about the same time in the majority of individuals (Szczeklik et al, 2001).
Although several studies show that ASA and other NSAIDs cause acute asthma crises in many patients, there is still no satisfactory diagnostic test for clinical use. Skin tests with aspirin derivatives (salicylate products) and serological tests are not successful in detecting aspirin induced asthma. This highlights the importance of an early and correct diagnosis of aspirin-sensitivity in order to avoid any unpredictable complications in susceptible individuals.

At present the accepted method for determining sensitivity is by challenge testing. There are four, main forms of aspirin challenge, depending on the route of aspirin administration: oral, bronchial (inhaled), nasal and intravenous tests. None so far has been demonstrated as suitable for clinical practice. Oral challenge is the most commonly used technique. However, it can provoke significant asthma attacks as well as being time consuming, (it often takes 2-3 days) which is why it has been restricted predominantly to research work on aspirin sensitivity (Casadevall et al, 2000, Pawlowicz et al, 1991).

Aspirin sensitivity has been considered in several studies as a type I hypersensitivity reaction that may be defined as a rapidly developing immunogenic reaction. It may occur as a systemic disorder or as local reaction. Firstly, a systematic reaction usually follows an intravenous injection of an antigen to which the host has already become sensitized and within minutes. Secondly, a local reaction that depends on the portal of entry of the allergen and may take the form of localized cutaneous swellings (skin allergy, hives), nasal and conjunctival discharge (allergic rhinitis and conjunctivitis), or allergic gastroenteritis (food allergy) (Cotran et al, 1999).

Aspirin produces an adverse reaction in several organs. Apart from the respiratory tree, the nose and the skin are frequently involved. These organs may be
simultaneously affected. The nose is the primary entry for inhaled air and allergens therefore it is the first region of the respiratory tract in contact with airborne pollution.

5.1.1 The Nose

The nose provides the first line of defence in the respiratory system. Its functions include clearance system and an inflammatory response which mediates mucosal swelling, immunological reactions and the exudation of plasma (Figure 5.1). The inflammatory response contains leukocytes that are capable of chemotaxis and phagocytosis. It also acts as a conditioning system to warm, humidify and filter inspired air. In addition, the nose is the segment of the upper airway that can be most accessible for morphological and pathological evaluation, since the same disease may, affect both nose and bronchi (Togias 1999, Raulf-Heimsoth et al, 2000).

A nasal challenge test involves the administration of suspected allergens directly into the nose in order to determine the nasal sensitivity of a tested substance. Although nasal challenge tests have been used for research purposes their clinical usefulness remains to be evaluated. (Raulf-Heimsoth et al, 2000).

5.1.2 Eosinophil

It is not clear whether the mechanism of chronic airway inflammation, in aspirin sensitive patients, is different from that in aspirin tolerant patients. Several studies demonstrated that intra-nasal application of L-ASA induces rapid recruitment of eosinophils into the nasal secretion in ASA sensitive, but not in ASA tolerant patients (Kowalski 1996), although no convincing evidence for the activation of these cells during ASA-induced reaction have been detected (Kowalski 1996, Szczeklik 1992).
Eosinophils are a prominent feature of aspirin sensitive rhinosinusitis, because of their abundance in nasal polyps, it seems that eosinophils are the primary cells that are regulating and perpetuating inflammation (Giembycz and Lindsay, 1999, Kowalski 2000). In healthy subjects the normal range of the absolute eosinophil count is 0.450/μL in whole blood, but the number is elevated with allergic disease and parasitic infections (Matthews et al, 1998, Heinecke 2000). Activated eosinophils are one of the most important cell types found in the nasal mucosa during immune responses, and the bronchoalveolar lavage fluid of subjects with ASA as compared with healthy volunteers (Sousa et al, 1997).

They are a source of inflammatory mediators, with a high capacity for production from their eosinophilic granules. These mediators include major basic protein (MBP),
plus eosinophil cationic proteins (ECP), peroxidase, arylsulphatase B, phospholipase D and histaminase, and cytokines such as IL-4 and IL-5 (Wu et al. 2000, Sampson 2001, Kowalski et al. 1996). The mechanism for eosinophil recruitment is not fully explained, but several studies showed that these cells are triggered by specific stimuli, such as IL-4, IL-5 and eotaxin (Fernvik et al. 1999).

5.1.3 Drug Absorption

Absorption is the passage of the drug from the site of administration into the plasma, before it reaches its site of action. The absorption of the drug is not required for the drug to act in the body. The main routes of administration are:

- Sublingual
- Oral
- Rectal
- Application to epithelial surfaces (e.g. skin, cornea, and Nasal mucosa).
- Inhalation
- Injection.

When the effects of the drug need to be locally studied, cutaneous administration is mainly used. Most drugs including those that are strong acids are generally absorbed very poorly through the skin (unbroken) due to their low liquid solubility. L-ASA was absorbed and observed in most of the patient’s serum and was measured. The amount of the absorption was determined by total serum, the patient’s blood was collected twice, before and after the administration of the L-ASA.
5.1.4 Rhinomanometry

Rhinomanometry is a system used in estimating the response to allergy medications, pharmacological challenge, and to environmental pollutants. The earliest account of any objective measure of nasal airflow was by Zwaardemaker in 1889, who placed a cold mirror beneath the nostrils and measured the size of the condensed breathing spots that were produced. This device is better known as Glatzel’s plate after the modifications and refinements he made to it (Naito 1997).

Pressure can be measured from one nostril, known as anterior rhinomanometry or from the nasopharynx, known as posterior rhinomanometry. In this study the posterior method was used. Posterior rhinomanometry is a simple and non-invasive physiologic test. The subject holds a fitted mask, which is firmly placed with a thin plastic sensor tube between the lips while breathing through the nose. Flow and pressure sensors register the amount of air per second in the nose during inspiration and expiration and the corresponding pressure in the airways (Figure 5.2). The registered values are presented using a graphically based software application, and the pressure-flow curve measurements of total NAR were made at a sample pressure of 75pa. The method was programmed to give the mean for two consecutive measurements of nasal airway resistance for each nostril, and measurements were made of five consecutive breaths (Figure 5.3).
Figure 5.3: Typical x/y plot for nasal pressure and flow

In order to calculate nasal resistance the nasal airflow is measured at sample pressure of 75 pa. The nasal resistance varies with the slope of the curve according to each individual airflow.
5.1.5 Aims

- To detect the nasal sensitivity of patients who have a response to aspirin or salicylate and to investigate whether the response of nasal hypersensitivity during local aspirin challenge tests, and allergy in general, are associated.
- To determine whether it is possible to use rhinomanometry as a diagnostic method for detecting aspirin sensitive patients.
- To explore the hypothesis that eosinophils contribute to IL-4 production in ASA airway secretion. Nasal lavage (NAL) was collected to examine the eosinophil production of these cytokine after exposing the area to specific L-ASA and compared the results of patients who are aspirin sensitive and atopic patients (Sousa et al, 1997, Gemou-Engesaeth et al, 2002).
5.2 Methods

5.2.1 Patients

Forty-seven subjects aged 18 – 74 years were studied by nasal challenge. Out of these, 28 were aspirin-sensitive, who reported adverse reactions after ingestion of ASA and other NSAIDs, some of them documented by emergency admission to hospital. 23 out of 47 were asthmatic, 11 had urticaria.

These subjects were distributed into four groups according to their response to aspirin administration, 18 atopic aspirin-sensitive individuals (6 female and 12 male) 10 atopic non-aspirin-sensitive (4 female, 6 male) 10 non-atopic aspirin-sensitive individuals (9 female, 1 male) and the control group consisting of nine healthy volunteers (7 female, 2 male) with no history of aspirin sensitivity.

No cyclooxygenase inhibitors and antihistamine therapy nor topical steroidal were used for 48 hrs prior to the nasal challenge.

The local Ethics committee approved the study protocol. Written informed consent to the performance of nasal challenge testing was obtained from all participants. Thirty-one of them were currently non-cigarette smokers and 13 were occasionally smokers, 7 out of 31 were ex-smokers of more than 20 to 35 years.

The aspirin challenge was carried out under the supervision of Dr Mehta, at Llandough hospital.

5.2.2 Materials

Chemicals used were lysine-acetylsaliclyc acid (Aspisol) provided by Bayer UK Ltd. The patients were challenged with saline and L-ASA solution at 3 different doses 10mg, 20mg, and 50mg. Fresh L-ASA solution was prepared each day immediately
before the start of the challenge by dissolving crystalline L-ASA in 0.9% sodium chloride. Aspirin administration was modified from the method described in previous study (Pawlowicz et al, 1991, Casadevall et al, 2000).

5.2.3 Set up of Rhinomanometry

The nasal resistance reading was taken twice to standardise it, since it is variable due to the way the patients handle the mask. In addition, each time a measurement was repeated all previous data was deleted from the computer, thus ensuring that the measured value was always the average of eight measures of nasal airways resistance (NAR).

The nostril with lower resting resistance was selected for the challenge. The initial exposure was a control solution of 100mg/ml of saline (Sodium Chloride) with pH 7.4 that was administrated locally into the nostril from a pipette tip. Consecutive doses of L-ASA solution were administrated every 15min and the forced expiratory value in one second (FEV\textsubscript{1}) was measured after each dose.

NAR was measured every 15min after each exposure and established by taking the mean of 5 measurements over 1 min, to estimate baseline resistance. After the last exposure, if the patient showed a positive response NAR, was monitored for a further 40min. A positive reaction was defined as a fall in FEV\textsubscript{1} of at least 20% below the patient’s baseline value or by an increase in NAR (200%) above the base line value.

The lack of bronchial response or any other symptoms in the following 2 hours from administrating the ASA was taken as an indication that the subject was not aspirin sensitive. Subjects were asked to score their symptoms of: cough, runny nose, blocked nose and sore throat. These symptoms were related on the following scale:
1. Not present.
2. Mild
3. Moderate
4. Severe

Previous studies have shown that, by training patients using this technique from information presented in this graphical format, problems with each measurement could be quickly recognised and appropriate action taken immediately. The subjects were trained for 30 minutes to master the technique of posterior rhinomanometry. Those subjects who were unable to master the technique within the 30 minutes were excluded. Patients were asked to breathe at a normal rate and depth through the measurement. After the acquisition of data from the first five breaths, the measurement was then repeated for a second time, after a short rest period during which the mask was removed (Figure 5.9). Prior to each measurement of nasal airflow, the patients were asked to blow their nose gently to clear it from excess secretion.

5.2.4 Drug absorption in the tested individual

In order to check whether local administration of aspirin has a systemic action, blood was obtained and salicylate in serum was measured before and after the administration. Analysis of serum aspirin levels was performed by gas chromotography.
5.2.5 The measurement of nasal Lavage (NAL) in aspirin sensitive individuals

5.2.5.1 Patients
our females and one male, aged between 34 and 48 years. 3 out of 5 were healthy non-aspirin sensitive volunteers, 2 were atopic aspirin sensitive. None of them were on corticosteroid or cyclooxygenase inhibitor medication, during the procedure. All of them were informed about the procedure, and consent for their participation was obtained. The ethical committee of the university approved the study protocol.

5.2.5.2 Lavage protocol

Lysine aspirin (Aspisol; Bayer AG, Germany) was diluted freshly each day in saline to produce a 1 M (0.1M=18mg/ml of ASA) stock solution, and then it was diluted to reach 9mg/ml concentration that was used.

The subjects were seated with their heads flexed about 30° forward. Washing of the nose and lavage collection were performed immediately after 5 min exposure to Saline (0.9%NaCl) as baseline, and 5 min after L-ASA from the administration. A syringe (10ml) attached to a soft rubber catheter to which a nasal olive was adapted for close nostril fitting was filled with 5ml saline and inserted into the nasal cavity. All washes were performed on one side of the nose only.

The lavage fluid was flushed slowly into the nasal cavity and back into the syringe five times. The procedure was then repeated with 5ml L-ASA.

The volume of the lavage fluid recovered was transferred into polypropylene tube to be centrifuged at 400g for 10 min; the cell pellets were resuspended in 2ml of supernatant. The samples were kept on ice for 30 min. The method was modified from previous studies (Roponen et al, 2001, Raulf-Heimsoth et al, 2000).
5.2.5.3 Preparation of the slides for cytospin

All the slides were degreased by immersion in 100% ethanol for 10min. The alcohol was shaken off and immersed in 0.01% polylysine solution for 30min. The solution was rinsed off with distilled water and allowed to dry. The coated slides were stored in a dust free container until use.

A cytofunnel chamber with filter card (Cytospin® Cytocentrifuge) was used. The funnel optimises cell yield, and decreases the possibility of cross-contamination between the samples. 100µl (NAL) was placed from each sample into the funnel separately (Figure 5.5-B). The slides were placed into the cytocentrifuge and spun at 32x g for 10min (Figure 5.5-A).

5.2.5.4 Esosinophil count

Collected cells were used for a differential cell count. After the slides were prepared by cytospin, they were fixed and stained with May-Grünwald-Giemsa method; slides were evaluated by light microscopy under oil immersion. Total cell counts were performed using a haemocytometer with improved neubauer ruling.

5.2.5.5 IL-4 ELISA

The supernatants of nasal lavage were used for the measurement of inflammatory mediators like IL-4 in the culture supernatants. ELISA kits (Quantikine® HS, R&D Systems Europe Ltd) according to the manufacturer’s instructions. The assay sensitivity and the lower detection limit for IL-4 were 0.25pg/ml.
5.2.6 Statistical Analysis

The results throughout are presented as the mean value ± standard error (SEM). Differences in data were analysed using one-way analysis of variance (ANOVA) and paired t-test. Data was analysed using the Minitab statistical program (version 12.1).
Figure 5.4: Patient undergoing Posterior Rhinomanometry.
Figure 5.5: (A) Cytospin centrifuge - (B) Cytofunnel
5.3 Results

5.3.1 Nasal challenge with L-ASA

It was possible to obtain satisfactory results from about 80% of the subjects challenged with aspirin using rhinomanometry (Figure 5.6).

In control (non-atopic) group:

None of the nine tested subjects experienced any clinical deterioration during the procedure. No significant change in NAR or FEV₁ was observed (as expected) (Figure 5.8).

Atopic aspirin-sensitive group:

In four patients, the administration of 20mg L-ASA induced extensive itchy skin and coughing during the nasal challenge, at which point no more aspirin dose was administered. One aspirin-sensitive patient showed a significant increase in NAR 398%, this rise was accelerated with the amount of L-ASA (Figure 5.7). Most of the symptoms typically appeared 30-60min after the first dose. After the last dose of aspirin, two patients developed chest tightness. In one of these symptoms lasted until late evening and the other was admitted to hospital. Seven patients out of eighteen did not respond to the aspirin challenge. The majority of patients in this group had significant response to the aspirin challenge.

Non-atopic aspirin-sensitive group:

Four subjects showed a slight increase in the NAR after the last dose of aspirin. One of these patients, a female, showed a significant increase of 357% NAR after the last dose of aspirin 50mg (Figure 5.9). The challenge was discontinued for two female patients after the second dose 20mg. One showed tightness in the
Figure 5.6: The variation of the total NAR for each of the 47 subjects, measured every 15min over four-interval measurements.

Each bar represents each subject. ● represents the Atopic subjects with aspirin sensitivity. ● represents the Non-atopic subjects with aspirin sensitively. The 2 sets remaining represent the Control groups; ● Healthy subjects. ● Atopic non-aspirin sensitive. Subjects 13, 21, 30 had zero flow for the duration of the investigation. 23% of the subjects had NAR below zero.
chest and red eyes, the second showed itchy skin and coughing. A female patient (68 years old) showed a significant decrease – 272 % NAR, with much release of nose blockage after the challenge.

**Atopic non-aspirin sensitive group:**

Four out ten tested subjects showed slightly increased NAR. These rises were not statistically significant. One subject out of nine had runny eyes after the second dose of aspirin 20mg, and showed an increase in NAR of 140%.

Most of the patients who showed an increase in NAR were females between 46-65 yrs of age, since the symptoms of the disease emerge earlier in females than males; previous studies (Szczeklik et al, 2000) have shown similar results. In all groups, no patient developed a fall in FEV$_1$ > 20%, except for two who showed a drop of 29% - 42% and both were atopic aspirin sensitive (Figure 5.8).

Asthma was the most common symptom of all the subjects studied (23 out of 48 were asthmatic), followed by Urticaria (11 subjects out of 28 were aspirin-sensitive). This was expected as most aspirin sensitive patients have this condition. The atopic aspirin sensitive subjects showed a significant increase in the NAR compared with the other 3 groups, the rise was accelerated with the amount of L-ASA. However four non-atopic aspirin sensitive subjects showed a slight increase in the NAR with the increase of the aspirin administration dose during the last dose (Figure 5.9)

Out of 28 patients with proven aspirin sensitivity, 19 of the tested subjects came from families with a history of asthma (1-2 members being asthmatic or allergic). However, there were only 6 cases of familial intolerance to aspirin. Four who were asthmatic and aspirin sensitive had fathers with similar symptoms, but no tests had been performed on them.
One 18 year old male with a 59 year old father, presented a history of angioedema after aspirin ingestion, this was confirmed in a nasal challenge test with 183% change in NAR from the baseline for the son, while the father showed a negative response. The response to the challenge was marked by a moderate rash on the face, neck and upper trunk. Another patient was a 56 year old asthmatic aspirin sensitive male whose father had urticaria and coughed after aspirin ingestion. The patient showed 209% in his NAR as a response to the nasal challenge (Figure 5.8).

There was no difference in the response to saline between the 4 groups observed. In contrast, a significant difference in response to the aspirin challenge (p=0.056) was observed in the aspirin sensitive subjects (atopic or non atopic) (the mean was 0.33 baseline and 0.68 after the challenge). On the other hand, in the control groups there was no statistically significant change in the NAR reading before and after the challenge with aspirin (p=0.20). There was a statistically significant increase in the NAR value when the two groups (aspirin sensitive and non aspirin sensitive) were compared (p=0.004)(Figure 5.10).

Interestingly, one of the unexpected outcomes, nothing similar in the literature, was that 11 out of 48 tested subjects, who showed negative response to the aspirin challenge, experienced a decrease in nasal congestion after the administration of L-ASA.
Figure 5.7: The effects of aspirin in the NAR for a patient during the aspirin challenge.

Two curves are shown at this figure. The blue curve represents the first reading after the administration of 20ml freshly prepared L-ASA. The pink curve represents the second reading after the administration of 50ml L-ASA.
Figure 5.8: The effects of L-ASA (Lysine acetyl salicylic acid) doses on the forced expiratory volume in first second (FEV\textsubscript{1}) during the nasal challenge on the four studied groups.

<table>
<thead>
<tr>
<th>Dose of L-ASA mg/ml</th>
<th>untreated</th>
<th>PBS</th>
<th>10mg/ml</th>
<th>20mg/ml</th>
<th>50mg/ml</th>
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<td>atopic-non-AS</td>
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<td>non-atopic</td>
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The four studied groups are represented in four different colors. AS: aspirin sensitive. Doses shown are 10mg/ml 20mg/ml and 50mg/ml. Maximum cumulative L-ASA dose was 80mg/ml. Control volunteers (Non atopic) and atopic non-aspirin sensitive (atopic-non-AS) showed no fall in FEV\textsubscript{1}cc after the challenge. While the atopic aspirin sensitive group (atopic-AS) and non-atopic aspirin sensitive group (non-atopic/As) showed a falling in FEV\textsubscript{1}cc after the administration of L-ASA. PBS was used as negative control. Error bars represent...
Figure 5.9: The effect of L-acetyl salicylic acid (L-ASA) on nasal airways resistance on the four studied groups

The aspirin sensitive groups (atopic aspirin sensitive and non-atopic aspirin sensitive) showed a high response to L-ASA challenge compared with the other 2 groups. Atopic aspirin sensitive group showed a significant increased in NAR % as the L-ASA dose increased. Error bars represent the ± SEM.
Figure 5.10: The effects of L-acetylsalicylate acid (L-ASA) on the 2 sets of Aspirin-sensitive and non-aspirin sensitive subjects

The histogram illustrates the amount of change in NAR value in the main two groups aspirin sensitive and non-aspirin sensitive, before and after the challenge with L-ASA. Bars represent the SD error of 47 subjects. The dosage of saline was 100μl.
An 8.5mg/ml L-ASA was absorbed after the administration of 80mg/ml of L-ASA as determined by total serum. This represents approximately 11% of the active substance.

Age had an effect in the patient’s response to the L-ASA challenge. Out of 26 females, 4 patients showed high NAR%, these patients shared two characteristics; firstly, all of them were over 45 years old. Secondly, they were all smokers (although one is a social smoker and the other three gave up over 30 years ago). The highest reading of NAR % obtained from this experiment in a tested female (63 years old) was 357%. Out of 21 males, 4 showed high NAR% (112%, 183%, 209%, 398%). The highest reading obtained was for a 58 years patient old who was an ex-smoker (who stopped smoking 30 years ago) he was in the atopic aspirin-sensitive group (Figure 5-11).

Figure 5.11: Age and L-ASA challenge.

The figure illustrates the correlation between age and the positive responsive to the aspirin nasal challenge. Old patient showed high responce to L-ASA, and showed high NAR % compared with the younger patient one.
5.3.2 Nasal Lavage

5.3.2.1 Eosinophil cell count

Eosinophil infiltration in airways after challenge is a consistent feature of atopic asthma. The cell population obtained in this study consisted mostly squamous epithelial cells 94% and small very few leucocytes predominantly of neutrophils.

One must take into account that eosinophils are generally low. However, the leucocyte count for all subjects never exceeded 2 cells, only one eosinophil was seen from all the slides examined.

It was decided that the number of cells obtained from these samples were too low to use for further investigation.

5.3.2.2 Cytokine analysis (IL-4)

All lavage samples had such low protein content that none had a cytokine level within the limit of detection of the assays need. This remained the case even after concentration by up to 10 times, no further investigation carry on.
5.4 Discussion

There have been many attempts to find a more sensitive, rapid and specific technique for determining aspirin sensitivity with less risk to the subject. The inhalatory nasal challenge with L-ASA was first introduced by Bianco 2000. His procedure was faster and carried less risk than the oral challenge tests. Other investigators have used nasal challenge testing on similar group (Llamazares et al, 1999). Ours is the first to apply a nasal challenge to these 4 groups (atopic non-aspirin sensitive, atopic aspirin sensitive, non-atopic aspirin sensitive and healthy volunteers (negative control), to measure objectively the effects of nasal aspirin administration on nasal responses using Posterior Rhinomanometry. In addition, contrary to previously published reports where challenge testing was performed over two days, this study was run for 3 hours, and the tested individuals were able to leave after an assessment was made of nasal airways resistant (NAR) and lung function.

The technique has less risk compared with oral and bronchial challenge testing. Although oral and bronchial challenge has similar specificity, the oral test has greater sensitivity. However; it involves a greater risk as it may cause bronchospasm crisis in asthmatic patients (Szczeklik et al, 2001, Llamazares et al, 1999). In oral challenge tests with ASA the systematic reaction is much higher, observed with the development of angioedema, skin rash, and asthmatic crises an anaphylactic reaction.

A study done by Pawlowicz and his co-workers in 1991, used rhinomanometry (passive anterior) in the studied groups. Our study, posterior nasal challenge testing with ASA showed good evidence of sensitivity and specificity in the detection of aspirin sensitive patients. The total dose of lysine-aspirin ranged from 90 mg/ml to 182 mg/ml in several studies, while in our study the maximum cumulative L-ASA
dose was 80mg/ml. (Schaefer et al, 1999, Szczeklik et al, 2001)

The administration of ASA locally into the nostril, where the rate of absorption is quite low, entails less risk of severe reaction than the oral and bronchial challenge. The nasal challenge test with ASA is therefore a simple technique, which is safer and quicker than oral and bronchial challenge. The response observed is quicker and is mainly restricted to the airways and can be more easily controlled than those arising after oral challenging.

Several reports have implicated cigarette smoking as a likely contributing factor in the development of asthma. Smoking is known to affect the local cellular immunity, and this may make smokers more susceptible to develop this syndrome. From our data, the high reading of NAR has been shown mostly among smokers (previously smokers), compared with other patients who are non-smokers. A 3-times increased NAR reading in smokers was observed compared to non-smokers.

Another factor linked to increase the susceptibility to this syndrome is the age. Older patients react more to the nasal challenge than younger subjects. The highest reading of NAR was 357% and 398% were obtained from a female aged 63 years old and a male aged 58 years old. Both these patients were previously smokers. This agreed with several studies, that stated that aspirin induced asthma is a condition more commonly recognised in middle age females. Moreover, the drug metabolism and renal function are less efficient in the elderly; therefore, drugs may tend to produce greater and more prolonged effects in both cases.

The study clearly demonstrated that patients who had not received any anti-inflammatory treatment and had not followed a salicylate avoidance diet gave significantly better responses than patients receiving therapy (figure 5.6 patient No-
This suggests that the nasal challenge and rhinomanometry is a suitable diagnostic test for newly diagnosed patients rather than those in who therapy has been initiated.

In addition to the nasal challenge, nasal lavage (NAL) was performed to measure soluble factors in the fluid. However, this technique was not suitable enough to recover the required amount of eosinophil cells. This has also been an observation supported by other researchers (Raulf-Heimsoth et al, 2000, Roponen et al, 2001). It was therefore very difficult to monitor eosinophil cells and determine the soluble factors participating in their function such as cytokines due to the low number harvested in samples. The nasal lavage procedure was therefore stopped due to insufficient yield of cells.

5.4.1 Limitation of the Rhinomanometry

The use of rhinomanometry for analysis had a number of limitations. Some patients (6 out of 47 tested subjects) experienced discomfort and irritation when using the oral tube. The apparatus seems to be affected by any minor adjustments during the analysis such as moving the oral tube or changing the seating position, thus making data collection difficult to achieve and time consuming.

These difficulties made it impossible to perform this test on some of the individuals in the study.
Chapter six

General Discussion
6.1 General Discussion

Aspirin (acetyl salicylic acid) is the oldest and most widely used non-steroidal anti-inflammatory drug (NSAIDs). These agents have been used primarily for their anti-inflammatory, analgesic and antipyretic effects, although recently aspirin has been shown to be effective in the management and prevention of non-inflammatory conditions, including coronary and cerebral ischemia and gastrointestinal cancer (Cianferoni et al, 2000). However, prophylactic aspirin and NSAIDs are considered the second or third in the ranking of drugs producing idiosyncratic reactions. The mechanisms underlying the pseudoallergic drug-induced adverse reactions related to aspirin and related compounds have long been a matter of debate. The only certainty has always been the absence of any detectable immunologic mechanism (DeSwarte et al, 1997).

Many questions still remain unanswered, about aspirin allergic disorder, such as why some people are more susceptible to allergy following ingestion of certain types of food and drugs? This is still one of the major challenges in this field of research.

The diagnosis of aspirin sensitivity can usually be established by a full patient history. Skin tests are of no value in the diagnosis of ASA, NSAIDs or dietary salicylate sensitivity. Also, currently these are no reliable in vitro tests available for the detection of this type of sensitivity. The oral challenge test is the only definitive diagnostic test available at present. This test can only be performed by experienced physicians in a hospital setting because of the risk of severe reaction and the risk of developing acute asthma (DeSwarte et al, 1997).

The aim of this study was to develop definitive diagnostic tests that can be utilized in atopic and non-atopic patients with sensitivity to aspirin, other NSAIDs and
salicylates in foods. It was hoped that this work would enable the development of a reliable practical test to detect people with a high risk of developing symptoms after ingestion of aspirin, NSAIDs or salicylate in food.

Several approaches have been used in this study. (1) Flow cytometry was used to quantify the expression of CD63 in activated basophils between treated and untreated samples with L-ASA (chapter 2).

(2) ELISA was carried out in two stages to investigate PGF$_2\alpha$ displacement by aspirin in plasma albumin and to measure the IL-4 protein production on treated and untreated samples with L-ASA respectively (chapters 3, 4).

(3) Rhinomanometry was used to detect sensitive patients who have a response to local aspirin challenge (chapter 5).

(4) Molecular techniques RT-PCR were applied to detect the expression IL-4 mRNA level in the suspected subjects after treatment with L-ASA compared with allergic or healthy volunteer (chapter 4).

The following sections will summarise the main findings of this study.

6.1.1 Role of basophils and the expression of CD63

Activation of basophils by IgE cross-linking is thought to be one of the main mechanisms of atopy. CD63 is glycoprotein present in basophils as well as in other leukocytes and platelets. In resting basophil, CD63 exists in the granular membrane and is highly expressed on basophil membrane only after basophil activation (Moneret-Vautrin et al, 1999).
In this study using a flow cytometric method, with triple labelling fluorescence anti-IgE, anti-CD63 as well as anti-CD14, the activation of basophils were measured after stimulation with specific stimuli.

Data showed clearly that CD63 was very useful in detecting activated basophils from atopic subjects. The number of gated activated basophils per sample expressing CD63 ranged from 300-490 cells in the majority of the patients out of 1000 cells of total whole blood counted by the flow cytometry (figure 2.4-C). CD63 expression was up regulated in response to an allergen specific to an individual allergy, this statement agrees with the observation of (Monneret et al, 1999, Abuaf et al, 1999). These studies also showed that aspirin (L-ASA) also increased the expression of CD63 on the basophils membrane in the aspirin sensitive patients. Lysine-ASA had no effect on basophils for subjects with no history of aspirin or salicylate sensitivity whether or not they were atopic.

In general, the activation of the cells with FMLP showed high expression of CD63 in the four study groups (non-atopic, non-atopic aspirin sensitive, atopic aspirin sensitive and healthy volunteers) (figure 2.6-2.7), the result agrees with previously published reports (Knol et al, 1991).

The L-ASA dose has crucial effects on the expression of CD63, high doses of L-ASA (18 mg/ml) suppressed the expression of CD63, while at low doses (0.036 mg/ml) the expression was enhanced until it reached its optimum at (1.8 mg/ml) of L-ASA (figure 2.6-2.8). These findings comply with previous studies where it was also noticed that high doses suppress the expression of CD63 (Monneret et al, 1999, Abuaf et al,1999). Currently, there is no explanation for the overall decrease in expression of
CD63 at this concentration. This may raise the possibility that high concentrations are toxic to the cells and may lead to down regulation of CD63 expression.

The expression of CD63 in activated basophils after stimulation with L-ASA showed some variation in the aspirin sensitive patients. A high fluorescence intensity of CD63 response (340) was obtained from only a low number of activated basophils (102 cells) in some subjects. The presence of high CD63 intensity in 17 patients with low basophil number, together with low intensity in 9 patients with high basophil number could be explained as follows; firstly, it could be related to the difference in CD63 receptor numbers on the surface of basophils. Secondly, this probably refers to the cellular immune response mechanism of each individual (inter-individual variability) and the amounts of CD63 present in their cells. This variation in CD63 fluorescence intensity was unrelated to atopic state of the aspirin sensitive patients.

In order to validate this technique CD63 expression in basophils activated with other allergens (grass pollen) was also performed (figure 2.9). The results indicate that allergens other than aspirin can cause elevated expression of CD63. Therefore, it could be concluded that CD63 expression is not specific for aspirin sensitivity but can also be used to detect allergies, when aggravated by the causative agent.

**In conclusion**

CD63 detection by flow cytometry seems to be a reliable method for the detection of activated basophils stimulated by drugs e.g. L-ASA. In addition, flow cytometry may represent a fast and sensitive method for the diagnosis of aspirin and salicylate sensitivity using basophil activation.
6.1.2 The effects of aspirin on displacement of PGF$_2$α

Prostaglandins contribute to the pain associated with inflammation, as well they play a key role in the generation of fever. Several tissues and cells in human lungs produce prostaglandins, including bronchial muscle, epithelium and inflammatory cells. Aspirin and other NSAIDs cause bronchoconstriction in about 15% of asthmatic subjects, by inhibiting the COX synthesis, which leads to prostaglandin synthesis blocked (Bennett 2000). Therefore, the inhibition of these mediators has numerous effects in asthmatic patients (AIA). This work discusses the effects of aspirin on the release of PGF$_2$α, which has bound to plasma albumin in the aspirin sensitive group and compare it with healthy volunteers.

The findings show that the amount of PGF$_2$α obtained was high in both treated and untreated sera, in atopic and non-atopic subjects (up to 319 pg/ml) compared with the given standard. Aspirin did induce the release of PGF$_2$α from serum, however none of these changes reach statistical significance. No differences in the response to aspirin were found in the four tested groups. For example, there was no significant difference in the level of PGF$_2$α in aspirin sensitive patients and the healthy volunteers, after activating the patients’ serum with L-ASA for 45 min (figure 3.4).

Incubating the patients sera with aspirin between (1-4 hours) increased slightly the release of PGF$_2$α in the aspirin sensitive group, whereas in the atopic non-aspirin sensitive and the healthy volunteer groups no such increase was detected, although, this increase was not statistically significant (figure 3.5).

None of the four study groups showed any statistically significant difference in the amount of plasma PGF$_2$α level after the addition of aspirin. This implies that no
relationship exists between the length of incubation time and the amount of PGF$_2$$\alpha$ released. The fact that PGF$_2$$\alpha$ levels in the control group showed no difference in PGF$_2$$\alpha$ when compared with the other 3 groups may relate to the possibility that although the control group was comprised of healthy volunteers, we cannot exclude the possibility that they may have other conditions that may contribute to the production of higher amount of PGF$_2$$\alpha$ than would be expected in normal individuals.

**In conclusion**

The results of this work does not support the presumed increased of PGF$_2$$\alpha$ in aspirin sensitive patients as suggested in previous studies (Williams et al, 1991, Plaza et al, 1995), but agrees with (Bennett, 2000) findings that aspirin has no effect on the reduction of PGF$_2$$\alpha$ synthesis in aspirin induced asthma (AIA). This implies that the measuring of plasma PGF$_2$$\alpha$ level alone in aspirin sensitive patients is not a suitable test to predict or detect the group.

**6.1. 3 Production of cytokine from aspirin sensitive patients**

Several studies suggest that the reaction to aspirin is IgE- mediated (Lee 1993, Vervloet and Durham, 1998, Wedi et al, 2000). Therefore, it was useful to detect IL-4 in the aspirin sensitive group and compare it with the non-aspirin atopic patients. This is because IL-4 is produced in type I hypersensitivity, which is fundamental to the pathogenesis of allergy causing IgE switching in B-cells and mast cells proliferation (Humbert et al, 1997).

ELISA was used to detect IL-4 protein in cells free supernatant from lymphocyte cultures from aspirin sensitive subjects and compare it with atopic non-aspirin sensitive individuals. The result showed that phytohemagglutinin (PHA)(100$\mu$g/ml)
used as a positive stimuli boosted the release of IL-4 protein in all groups; it was significantly higher (p<0.05) compared with samples treated with grass pollen (figure 4.2B).

IL-4 protein was detected in all the tested samples stimulated with grass pollen for 30min at 37°C. There was a significant increase (p=0.05) in IL-4 levels releases in atopic grass pollen sensitive subjects compared with control volunteers, after stimulation the samples with grass pollen. However, the aspirin sensitive subjects showed no increase in the IL-4 levels between treated and untreated samples with aspirin (L-ASA). The slight increase of IL-4 level observed at concentration 1.8 mg/ml of LASA concentration in the two tested groups (treated and untreated), however it did not reach a significant level.

RT-PCR is still one of the most powerful molecular techniques available to examine differential gene expression between different cell types. However, some difficulties were encountered when using this technique to detect IL-4 mRNA. These include: (1) low amount of total RNA (<0.045ug/ml) yields from blood samples.(2) Contaminating DNA. (3) Inability to get an amplification product for samples treated with positive stimulants for IL-4 mRNA. Several different primer pairs were used together with different cycling parameters, yet no IL-4 mRNA was detected. The lack of IL-4 mRNA expression in the system, may have been due to the (1-3) hours incubation with ASA that was applied, which may not have been long enough to activate the expression of the IL-4 gene.

β-actin gene expressions were detected in all the samples indicating that RNA extraction, PCR reagents and electrophoresis were effective. Both frozen and fresh blood samples were used it this study. It was noted that the expression of β-actin was
stronger in the fresh samples compared to the frozen ones (figure 4.7). It is suggested that freezing could causes some degree of degradation; this may explain the lower expression in the frozen samples. The presence of β-actin bands in all the samples confirmed the previous conclusion regarding the low expression level of IL-4, and excludes the possibility of lack of expression due to degradation.

RT-PCR technique was used because it is a quick and easy method that involved a low amount of blood sample obtained from the tested individual. In this situation, the RT-PCR was not a valid procedure, and due to the limited time available for research, and repeated patient involvement, there were no further investigations. Future work in this area could include using a more sensitive approach such as the nested PCR technique, which could be useful in detecting IL-4 mRNA in aspirin sensitive groups.

**In conclusion**

The failure of L-ASA to increase IL-4 production in lymphocytes culture compared to the effect of specific allergen (grass pollen) suggests that IL-4 production plays no role in aspirin and salicylate sensitivity. The failure to detect IL-4 mRNA may be related to inadequate optimisation of PCR conditions. However, as all the investigations were carried out using patient samples it was felt inappropriate to continue to ask patients to return to clinic to allow further blood samples to be obtained for procedure which was unlikely to yield any significant data.

**6.1.4 Local affects for aspirin in allergic patients and Nasal lavage**

The effects of L-ASA administration on nasal responses using Posterior Rhinomanometry was assessed in 4 different groups (control, atopic non-aspirin sensitive, non-atopic aspirin sensitive and atopic aspirin sensitive).
Data showed statistically significant change in nasal airway resistance (NAR) between the 4 groups (figure 5.10). The aspirin nasal challenge was tolerated well by the tested subjects, and the test was simple, safe and quick, the experiment ran for 3 hours for each patients.

The lack of systematic symptoms seen in the aspirin sensitive subjects may be attributed to the low amount of the dose that was administrated into the tested subjects, the maximum dose was 80mg/ml. None of the tested healthy volunteers showed any complications during the procedure or after. In addition, in all tested subjects, no patient developed a fall in forced expiratory volume in the first second (FEV₁) of more than 20%, except for two who were atopic aspirin sensitive and showed 29% -42 % fall.

In total, only 3 out of 47 patients showed severe reaction after the challenge. This shows that the aspirin nasal challenge entails less risk and can be used safely in these patients. The reactions reported vary between chest tightness and red eyes (atopic aspirin sensitive) and 357% NAR after the administration of 50mg/ml. Another patient (non-atopic aspirin sensitive) developed itchy skin and coughing after the second dose 20mg with 140% NAR, both patients were females.

Most patients who showed a positive reaction to L-ASA challenge with high NAR (357%- female) were smokers or previously smokers. The NAR was 3-fold greater in these patients compared with the non-smoker results, not shown.

Age appears to have an effect in the subject’s response to drug challenge. The positive response of older patients to aspirin nasal challenge was significantly greater than youngest subjects, 4 females out of 26 showed high NAR%. A femal showed 357% NAR was aged 63 years old, and 398% for a male of 58 years old after
challenge them with L-ASA. Taken together, both these patients were previously smokers (figure 5.11). This observation confirms previous study that age and smoking has effects in drug challenges.

The response to saline challenge was constant in four tested groups, no statistical difference was observed. In contrast a significant difference in response to aspirin challenge (p=0.04) was observed in the aspirin sensitive subjects (atopic or non atopic) [(the mean was 0.33± SD (baseline) and 0.68± SD (after the challenge)]. On the other hand, the control subjects showed no statistically significant change in the NAR reading before and after the challenge with aspirin (p=0.20). There was a significant difference in the NAR value when the aspirin sensitive group and non-aspirin sensitive group were compared, (p=0.004)(Figure 5.10).

Interestingly, patients who had not received any medications such as anti-histamine and had not followed a salicylate avoidance diet gave significantly better responses than patients receiving therapy. This suggests that nasal challenge with rhinomanometry is a suitable diagnostic test for newly diagnosed patients rather than those in whom therapy has been initiated. In addition, one of the most surprising observations was 12 out of 48 tested subjects who showed no response to the aspirin challenge experienced a relief of nose blockage after the challenge with aspirin. One of them a 68 year old female showed a significant decrease -272% NAR, with relief in breathing (figure 5.6).

Nasal lavage is amongst the most utilised techniques to detect inflammatory mediators in asthmatic patients, since experimental studies have shown that inflammatory response in the nose may be predictive for the situation in a patient’s lung. Several studies show that the most important cell types found in the nasal
mucosa during inflammation are eosinophils that appear to be a prominent feature of airway inflammation in allergic rhinitis nasal polyposis, and asthma. Thus, these studies have shown an increased number of activated eosinophils in allergic patients, especially asthmatics (Petersen et al, 2001, Chanez et al, 1999). This study tried to compare the number of eosinophils and the concentrations of intracellular inflammatory mediators in nasal lavage (NAL) collected from aspirin sensitive and non-aspirin sensitive subjects exposed to local stimulation with L-ASA. However, because of the low numbers of cells isolated and the predominance of epithelial cells the study failed to obtain a satisfactory number of leucocytes and only one eosinophil was seen in all of the study subjects. Thus, the study was discontinued.

In conclusion:

This study suggests that aspirin sensitivity and salicylate sensitivity are not allergic responses, as they do not involve IL-4 production by lymphocytes. Basophils are strongly implicated in the pathogenesis of this disorder and probably in combination with mast cells. This study also suggests that the determination of CD63 expression in the presence of aspirin is a useful *in vitro* diagnostic test for aspirin and salicylate sensitivity and that together with an appropriate history may be sufficient to form a definitive diagnosis. Nasal challenge by nasal airways resistance test (NART) may prove useful in confirming diagnosis in newly selected patients in clinic. It seems that aspirin and salicylate sensitivity does not involve IgE-mediated mechanisms and as such is not an allergic response. However, it seems to involve an early activation of the same effector cells such as basophils and mast cells and eosinophils, they are of lipid mediators source that may be responsible for pathogenesis of aspirin induced asthma, which is supported by several studies.
Further Work

- Further studies will be needed to identify the specific cellular sources of allergic response mediators and their specific interaction. These studies could include the evaluation of cytokine production in this syndrome. This would require the recruitment of a larger number of aspirin sensitive patients.

- Further studies are required to combine PGF$_2\alpha$ determination with other markers which have been suggested to be markers of type I immediate allergy, such as measuring tryptase levels in mast cells and eosinophils before and following aspirin ingestion in ASA subjects (Sousa et al, 1997).

- In addition other factors such as the genetic factors, inter-individual variability in drug metabolism, combined with immunological background should be considered as underlying mechanisms.
Appendix
Appendix

Abstracts presented:

1- The effect of LD-lysine-mono-acetylsalicylate on Basophils CD63 expression in vitro.

This abstract was accepted and presented in the following conference, British Society for Allergy and Clinical Immunology, Annual Meeting July 9th –11th 2001.at the East Midlands Conference Centre Nottingham.

2- The efficiency of nasal challenge with aspirin using Rhinomanometry.

Appendix 2

Preparation of solution and reagents used in application of techniques described in this work.

- L-Acetyl salicylic acid (Aspirin) concentrations:

  The powder is 0.9gm + H₂O 5ml
  1.8gm ---------10ml H₂O
  1.8gm= 180mg in 1000ml

  The main Conc. Is 180mg = 0.9g/5ml = 1.8g/10ml
  1/10 = 18mg/ml
  1/20 = 9mg/ml
  1/50 = 3.6mg/ml
  1/100 = 1.8mg/ml
  1/500 = 0.36mg/ml
  1/1000=0.18mg/ml

- FMLP preparation:

  The product was obtained from the (sigma chemical Co.), it was dissolved in dimethylsulfoxide and was stored at -20 °C. to dilute it to be use for the experiment.

  20 μl FMLP + 1980 μl PBS. (FMLP=0.5ng/ml)
Figure 3.3: This standard curve demonstrates the mean absorbance for Optical Density and %B/Bo, against the concentration on a logarithmic X-axis, to calculate the concentration of PGF$_2\alpha$. 
**RT-PCR**

- **10x MOPS**

MOPS 42gm/lit 10.5g/250ml  
Sodium 6.8gm/lit 1.7g/250ml  
Acetate 4.1gm/lit 1.0gm/250ml  
EDTA 3.72gm/lit 0.93gm/250ml

Dissolve in 1liter litre RNAase free H₂O autoclave and store at room temperature in dark.

- **Tris- Borate- EDTA buffer**

  TBE Buffer Gel electrophoresis

  SIGMA-ALDRICH CO

- **Loading Buffer DNA**

  Formamide 0.72ml  
  Formaldehyde (37%) 0.26ml  
  10x MOPS 0.16ml  
  H₂O 0.18ml  
  Glycerol (80%) 0.1ml  
  Bromophenol Blue 0.08ml

- **Gel Loading Solution**

  RNA Queous Ambion®

- **DNA Free**

  Ambion The RNA Company

- **DNA: Ultra Pure Agrose electrophoresis Grade**

  Life Technologies.

- **RNA Agarose Fisher Scientific.**
• Annealing temperature equation:

\[ T_m = (A+T) \times 2 + (G+C) \times 4 = T_m \]

\[ T_m - 5 = \text{annealing temperature}. \]
References
References


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