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AN EVALUATION OF THE ANTI-ALLERGIC PROPERTIES OF POTASSIUM HUMATE

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ABSTRACT

Although the anti-inflammatory properties of humate derived from peat, sapropeles and mumie have been described, no clinical studies has been done on the anti-inflammatory effects of humate derived from coal. Leonardite humate compared favourably with prednisolone in suppressing contact hypersensitivity in a rat model.

According to a report by the European Agency for the Evaluation of Medicinal Products on toxicity studies (Feb 1999), humic acids extracted from brown coal has no toxic effects on rats in a chronic study at oral dosages as high as 1g/kg BW, whereas the LD50 in rats, after oral administration of humic acids, has been reported to be greater than 11g/kg BW. This report has recently been confirmed by a separate study.

The objective of this study was to establish the safety and the therapeutic efficacy of oral potassium humate (Humiboost™) in reducing the signs and symptoms of hay fever in atopic patients during the grass pollen season. In this parallel double-blind placebo controlled phase II study potassium humate was randomly assigned, at a dosage of 1.8g in divided doses/day, to atopic patients (n = 40) presenting with acute symptoms of hay fever. The blood and nasal samples were used to determine the safety of/and the effects of potassium humate on basophil activation, cytokine levels and eosinophil migration. A skin prick test was used to determine its anti-allergic effects, whereas a symptom score scale was used to determine its effects on the symptoms of hay fever.

The clinical safety of Latrobe valley potassium humate™ was proven. This study confirmed with a significant decrease in the skin prick test results and eosinophil counts that this product definitely possesses anti-inflammatory as well as anti-allergic properties, but due to no significant differences with the total symptom score it is yet to be proven that it alleviates the symptoms of allergic rhinitis. No significant differences were observed with regard to the neutrophil adhesion assay thus suggesting that potassium humate has a more specific anti-allergic action. With regard to it's mechanism of action, there are promising results concerning its effect on

the recruitment and activation of eosinophils by decreasing the expression of IL-4, IL-5, IL-8 and IL-1β, although not reaching statistical significance.

Key words: Humic acid, allergic rhinitis, eosinophils, basophils, cytokines, neutrophils, inflammation.

OPSOMMING

Alhoewel die anti-inflammatoriese eienskappe van humaat, afkomstig van turf, sapropel en mummie reeds beskryf is, is daar nog geen kliniese studies op die anti-inflammatoriese effekte van humaat afkomstig van bruinkool gedoen nie. Volgens 'n vorige studie is bewys dat humaat afkomstig van leonardiet, effektief is in die onderdrukking van kontak hipersensitiwiteit in 'n rot model.

Volgens 'n verslag deur die "European Agency for the Evaluation of Medicinal Products on toxicity studies" (Feb 1999) het humien sure, wat uit bruin kool geekstraeer is, geen toksiese effekte tydens 'n chroniese studie op rotte gehad by orale doserings so hoog as 1g/kg liggaamsgewig. Volgens die verslag is die LD50 in rotte, na orale toediening van humien sure, groter as 11g/kg liggaamsgewig. Hierdie verslag is onlangs, in 'n apparte studie, bevestig.

Die doel van hierdie studie was om die veiligheid en terapeutiese effektiwiteit van kalium humaat (Humiboost®) vas te stel sowel as om die effektiwiteit daarvan te bepaal om die tekens en simptome van hooikoors in atopiese individue gedurende die gras en stuifmeel seisoen te verminder. In hierdie paralelle, dubbel-blind, placebo gekontroleerde fase II studie is kalium humaat lukraak, teen 'n dosering van 1.8g per dag, aan atopiese pasiente (n=40) met hooikoors simptome toegedien. Bloed en nasale monsters is gebruik om die veiligheid en/of effektiwiteit van kalium humaat op basofiel akivering, sitokien vlakke en eosinophil migrasie te bepaal. Verder is 'n vel-prik toets gebruik om humaat se anti-allegiese aktiwiteit te bepaal terwyl 'n simptoom-telling skaal gebruik was om sy effekte op die simptome van hooikoors te bepaal.

Die kliniese veiligheid van kalium humaat van die Latrobe vallei is in hierdie studie bewys. Hierdie studie het ook, deur middel van resultate verkry vanal die vel-prik toets en eosinofiel tellings, bevestig dat kalium humaat beslis anti-inflammatoriese sowel as ani-allergiese aktiwiteite besit. In die geval van die totale simptoom-telling was daar egter geen betekenisvolle verskille om te bewys dat dit die simptome van allergiese rinitis verbeter nie. Geen betekenisvolle verskille is verder waargeneem wat betref die neutrofiel-vashegtinseksperiment nie, wat voorstel dat kalium humaat

‘n meer spesifieke anti-allergiese aksie het. Wat betref die meganisme van aksie is belowende waarnemings gemaak wat voorstel dat die werwing en aktivering van eosinofiele veroorsaak word deur die afname van die uitdrukking van Il-4, Il-5, Il-8 en Il-1β, alhoewel dit nie statisties betekenisvol was nie.

Sleutelterme: Mumien suur, hooikoors, eosinofiele, basofiele, sitokiene, neutrofiele, en inflamasie.

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LIST OF ABBREVIATIONS

ALP	Alkaline phosphatase
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AR	Allergic rhinitis
BA	Basophils
BHK	Baby hamster kidney fibroblasts
BW	Body weight
CCR3	Chemokine complement receptor 3
CD	Cluster of differentiation
CRE	Creatinine
CR3	Complement Receptor 3
CRTH2	Chemoattractant on thymus helper cell 2
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNBF	2,4-dinitro-fluorobenzene
DP	Prostaglandin D receptor
ECP	Eosinophil cationic protein
EDTA	Ethylenediaminetetraacetic acid
EO	Eosinophils
EPX	Eosinophil protein X
FBI	Fluorescent bead immuno assay
FITC	Fluorescein isothyocyanate
g	Gram/Gravity
GGT	Gamma-glutamine transaminase
HBSS	Hanks balanced sodium solution
HCT	Haematocrit
HGB	Haemoglobin
HIV	Human immunodeficiency virus
ICAM	Intracellular adhesion molecule
IFN	Interferon
IgE	Immunoglobulin E
IgG	Immunoglobulin G

IL	Interleukin
K	Potassium
Kg	Kilogram
L	Litre
LD	Lethal dose
LT	Leukotriene
LY	Lymphocytes
MCH	Mean cell haemoglobin
MCHC	Mean cell haemoglobin concentration
MCV	Mean cell volume
µl	Microlitre
mg	Milligram
ml	Millilitre
mm	Millimetre
MO	Monocytes
MPO	Mieloperoxidase
NA	Sodium
NE	Neutrophils
NE	Neutrophil elastase
NF	Nuclear factor
nm	Nanometre
OPD	O-phenylenediamine
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PC	Phospotidylcholine
PE	Polyehylene
PGD	Prostaglandin
PLT	Platelets
PMA	Phorbol myristate acetate
PMNL	Polymorphonuclear leukocyte
RANTES	Regulated on activation normal T cell expressed and secreted
RBC	Red blood cells
rcf	Relative centrifugal field

RDB	Reagent dilution buffer
RDW	Red cell distribution width
rpm	Revolutions per minute
SD	Standard deviation
SPT	Skin prick test
Tc	Thymus cytotoxic cells
TGF	Tumour growth factor
Th	T helper lymphocyte
TNF	Tumour necrosis factor
Vfin	Final volume
vs	Versus
WBC	White blood cell

CHAPTER 1
LITERATURE REVIEW

1.1. Introduction

1.1.1. Allergic rhinitis

Allergic rhinitis is an inflammatory disease that is closely related to other allergic conditions and may be viewed as a local manifestation of a systemic immune disorder (Holgate, 1999). The immediate allergic responses are predominantly histamine mediated and explain symptoms such as watery rhinorrhoea, nasal or ocular itching and sneezing. The late responses are more complex and involves a systemic immune activation which then leads to symptoms such as nasal congestion, fatigue etc. This is mainly a Th2 type of reaction with typical expression of chemokines such as IL-4, 5, 6, 8 and 13, eotaxin and RANTES as well as the up regulation of adhesion molecules ICAM-1 and P-selectin (Elias *et al.,* 2003).

Successful therapy is not only to suppress the signs and symptoms of local manifestations of atopic diseases but also to address the basic immune deregulation in order to establish a more complete and permanent cure. To date, treatments consist mainly of topical or systemic antihistamines, glucocorticosteroids and decongestants. All these medicines are relatively expensive and are not without local or systemic side effects and furthermore do not address the abnormal immune response causally.

1.1.2. Eosinophils

Accumulation of tissue eosinophils is characteristic of allergic rhinitis, with eosinophils rarely being present within nasal mucosa of individuals who are non-atopic and have no nasal symptoms. Increases of eosinophils are evident both within the lamina propria and epithelium of the nasal mucosa (Breddin *et al*., 1993, 1995, Bentley *et al*., 1992, Jacobcon *et al*., 1999), and their recovery is also increased in nasal luminal samples (Andersson *et al*., 1989, Pipkorn *et al*., 1988, Salib *et al*., 2005).

During active allergic rhinitis, it appears that a combination of increased accumulation of tissue eosinophils and extensive degranulation produces high levels of extracellular deposition of eosinophil granule products in the target tissue. Comparative with these tissue changes, there are alterations of nasal luminal markers of eosinophil activation (Ahlstrom-Emanuelsson et al., 2004). One study using a nasal lavage technique in seasonal rhinitis demonstrated a 20-fold increase in the number of eosinophils in lavage fluid during the pollen season, which closely followed the pollen counts and symptom scores of the patients (Pipkorn et al., 1988).

Eosinophils are thought to mediate inflammatory and cytotoxic events associated with allergic disorders, including bronchial asthma, rhinitis and urticaria (Gleich, *et al*., 1993, Kroegel *et al*., 1994). This interpretation is in line with very established observations in which hypereosinophilic dysfunctions are clearly associated with tissue damage, in particular endomiocardial fibrosis (Wardlaw *et al*., 1995).

The eosinophil granule covers a spectrum of preformed highly toxic cationic proteins, including the major basic protein and eosinophil cationic protein, which are able to cause tissue damage by disrupting the membrane structure of target cells (Gleich *et al*., 1993). Eosinophils can also generate active substances like PAF, leukotriene C4, active species of oxygen and several cytokines which can lead to inflammation, bronchoconstriction and mucus hypersecretion, important components of the airway allergic dysfunction (Weller, 1994). The pro-inflammatory effector function of eosinophils has been supported by an extensive amount of studies accumulated throughout the 1980's.

1.1.3. Basophils

Research has provided insights into the possible role of basophils in allergic disease and immunity to pathogens. Most notably, the discovery that basophils rapidly produce large amounts of the regulatory cytokines interleukin IL-4 (Brunner *et al*., 1993; Arock *et al*., 1993) and IL-13, (Li *et al*., 1996; Gibbs *et al*., 1996; Ochensburger, 1996) together with the constitutive expression of CD40L (Yanagihara *et al*., 1998; Gauchat *et al*., 1993) and CCR3 (Uguccioni *et al*., 1997) on their surface, has fuelled speculations that extend beyond their recognized role as effector cells in IgE-mediated reactions.

Basophils have also been proposed to play a key role in allergy by directly inducing the switch to the IgE isotype in B cells independently of T cells. Basophils generated from human umbilical cord blood mononuclear cells, after cultivation in the presence of appropriate cytokines, expressed detectable levels of CD40L and induced IgG4 and IgE synthesis in B cells when stimulated with allergen. The induction of IgE synthesis was completely abrogated by neutralizing IL-4 and IL-13 with monoclonal antibodies or CD40L with soluble CD40 (Yanagihara *et al*., 1998). Therefore it is reasonable to assume that basophils, as a major source of IL-4 and IL-13 and by constitutively expressing CCR3 and CD40L on their surface, may play an important but underestimated role in the pathology of asthma and other allergic conditions.

1.1.4. Neutrophils

Neutrophils can contribute to the inflammation by releasing chemokines, superoxide anion and granular enzymes. Neutrophil elastase (NE) in fluids in the epithelial lining of the airways has been reported to destroy structures of the extracellular matrix, induce further inflammatory cytokine release (IL-1β, IL-6, IL-8, TNF-α, TGF-β), and produce clinical symptoms (Teran *et al*., 1997; Nadel *et al*., 1999; Gadek *et al*., 1979). Neutrophils can also produce a wide range of products including lipids (LTB4, PAF, TXA2, LTA4), proteases (elastase, collagenase, MMP-9), microbicidal products (lactoferrin, MPO, lysozyme), reactive oxygen intermediates (superoxide, H2O2, OH^{-}) and nitric oxide (Sampson, 2000).

These cytokines are likely to bring an additional neutrophil recruitment and hyper-responsiveness. However, because of their potential to release large amounts of histotoxic and other pro-inflammatory agents, this cell also has the capacity to produce significant tissue injury. Hence inappropriate or excessive activation of neutrophils *in vivo* has been implicated in the pathogenesis of a wide variety of inflammatory diseases (Brazil *et al*., 2005).

Neutrophils produce large amounts of LTB4 and also secrete its precursor LTA4. When the neutrophil is in apposition to airway structural cells, LTA4 may be converted either into LTB4 or into LTC4, a very potent bronchoconstrictor (Sala *et al*., 1997). LTB4 is a potent chemoattractant for neutrophils, eosinophils, monocytes

and fibrobroblasts. LTB4 also activates nuclear factor (NF)-kB (Stankova *et al*., 1992), promotes the synthesis of IL-5, IL-6 and IL-8 (Yamaoka *et al*., 1993), and enhances IgE synthesis in B cells (Yamaoka *et al*., 1994). Instillation of LTB4 into human airways leads to neutrophilia 4 h later (Martin *et al*., 1991). In addition, neutrophils are the first cell type to enter the lung following allergen challenge (Smith *et al*., 1992; Teran *et al*., 1995; Casale *et al*., 1997). Unlike eosinophils, the influx of neutrophils is transient following allergen challenge.

1.1.5. Cytokines

Th1 and Th2 cells antagonize each other, allergic inflammation results from the predominance of Th2-cytokines in relation to the Th1-cytokines (Romagnani, 2000). Thus infiltration of Th2 cells into the nasal mucosa has been described in allergic rhinitis (Yanagihara *et al*., 1998; Ying *et al*., 1994). According to the Th2 hypothesis in allergy Th2 cells promote IgE synthesis, whereas Th1 cells inhibit IgE synthesis. These opposing effects are caused by differential cytokine production. Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, but not interferon (IFN)-γ.

Th1 cells secrete IL-2, IFN-γ and tumour necrosis factor (TNF)-α, but not Th2-cytokines. The Th2-cytokines, IL-4, IL-5, IL-6 and IL-13, regulate IgE production and growth, whereas IL-4 and IL-5 regulates activation of eosinophils and IL-4, IL-9 and IL-10 regulates activation of mast cells and basophils. The local expression of Th1- and Th2-cytokines in allergic rhinitis has also been examined, with mixed results (Linden *et al*., 1995; Durham *et al*., 1996; Pawankar *et al*., 1996; Klimek *et al*., 1999; Benson *et al*., 2000).

In two studies of nasal fluids, IL-4/IFN-γ ratios were higher in school children with allergic rhinitis than in healthy controls (Benson *et al*., 1997, 2000). In further support of the Th2 hypothesis, Th2 cytokines were associated with the increased presence of eosinophils, ECP and IgE, but not neutrophils, in nasal biopsies and fluids from patients with allergic rhinitis (Ying *et al*., 1994; Benson *et al*., 1997, 2000; Cameron *et al*., 2000)

Th1- and Th2-cytokines can be secreted by other cells in the nasal mucosa, such as CD8+ T cells, NK cells, B cells, eosinophils, mast cells, basophils and epithelial cells (Varney *et al*., 1992; Salvi *et al*., 1999). Mast cells and basophils may release Th2-cytokines in direct response to an allergen (Cameron *et al*., 2000; Wilson *et al*., 2000). Nasal epithelial cells, neutrophils, mast cells, fibroblasts, for example, release eotaxin, IL-1β, IL-8 and TNF-α, which can induce influx and activation of eosinophils (Bradding *et al*., 1995; Abdelaziz *et al*., 1998; Terada *et al*., 2000).

Cytokine release from these cells can indirectly result from interactions with Th2 cells, e.g. nasal fibroblasts exposed to IL-4 and IL-13 secrete eotaxin (Terada *et al*., 2000). However, nasal epithelial cells may also release cytokines in direct response to proteolytic activity of allergens (Tomee *et al*., 1998). Moreover, epithelial responses occur quickly. Epithelial cells could therefore have a causal role in acute allergic inflammation, in addition to Th2 cells, mast cells and basophils.

Local elevations of IL-1β, IL-8 and TNF-α, have been described in some studies of allergic rhinitis (Sim *et a*l., 1994; Weido *et al*., 1996; Bradding *et al*., 1995; Bachert *et al*., 1995). Epithelial and other cells may also release immunosuppressive cytokines (Holgate, 2000). It is of note that at present more than 100 cytokines are described, and an unknown number of these may take part in regulating allergic inflammation.

1.1.6. Humic acids

Humic acids are macrocolloidal molecules that result from the decomposition of plants and are natural components of drinking water, peat, soil and brown coal. Mud baths, rich in humic and fulvic acids, were used for the treatment of various ailments, such as rheumatic conditions during the 19th century (Baatz, 1988; Lent, 1988; Kleinschmidt, 1988; Kovarik, 1988). Peat was also used during the First World War to treat wounds and amputations in field hospitals to prevent infections, relieve pain and facilitate healing (Haanel, 1924; Van Beneden, 1971)

There are currently several humic acid based topical and oral medications commercially available. Their therapeutic properties have been summarized by

Schepetkin *et al*., (2002) as antibacterial, antitoxic, anti-ulcerogenic, anti-arthritic, anti-allergic and anti-inflammatory. Studies by Kleinschmidt (1988) and Iubitska and Ivanov (1999) have shown that sodium humate therapy improves the clinical condition of osteoarthrosis patients (Soliev, 1983) possibly due to its anti-inflammatory and bone regenerating properties (Kelginbaev *et al*., 1973; Suleimanov, 1972).

Humic substances' beneficial effects have been partly ascribed to the ability of humic acids to absorb or chelate toxic compounds or metals (Stackhouse and Benson, 1989; Shanmukhappa and Neelaktan, 1990; Nifant'eva *et al*., 1999; Sauvant et al., 1999; Marx and Heumann, 1999) and to absorb xenobiotics (Nielsen *et al*., 1997; Prosen and Zupancic-Kralj, 2000), mutagens (Ferrara *et al*., 2000) and mycotoxins (Jansen van Rensburg *et al*., 2002). There are several mechanistic studies that were done on potassium humate, derived from bituminous coal, during the last few years. Van Rensburg *et al*., (1999 and 2000) and Jooné *et al*., (2003) found that this product stimulates lymphocyte proliferation by an increased production of the cytokine, IL2. This response was even more striking in the case of lymphocytes from HIV-infected individuals (Jooné *et al*., 2003) and was also observed *ex vivo* following administration of 4g of the product daily to HIV-positive individuals for two weeks.

It was found that potassium humate inhibits degranulation of inflammatory cells as well as the expression of CR3, a pro-inflammatory adhesion molecule, expressed by human neutrophils, leading to a decreased binding to ICAM 1, the ligand of CR3 present on inflamed endothelial cells (Jooné *et al*., 2001). Leonardite derived humate results in similar inhibition of CR3 expression (Data on file Department of Pharmacology, UP). The exposure of human neutrophils to this product resulted in decreased expression of CR3 by activated, but not resting cells, in a dose-related way. Inhibition of the expression of adhesion molecules by activated phagocytes, as well as the inhibition of the release of granule polypeptides, both of which are responsible for tissue damage during the inflammatory processes, are attractive targets for anti-inflammatory drugs.

Although the anti-inflammatory properties of humate derived from peat, sapropeles and mumie have been described, no clinical trials have been done on the anti-

inflammatory effects of humate derived from coal. Leonardite humate compared favourably with prednisolone in suppressing contact hypersensitivity in a rat model. In a study conducted by Van Rensburg *et al.,* (in press) it was determined that DNBF-induced contact hypersensitivity in rats was reduced with brown coal derived humate at a dosage of 61 mg/kg BW, which was administered by gavage directly after sensitization. The anti-inflammatory effects may have been due to either an inhibition of the sensitization or the inhibition of the specific challenge or both. There is also a possibility that humate adsorbs pro-inflammatory molecules such as cytokines and complement factors. In this same study it was also found that brown coal derived humate at a dosage of 1000 mg/kg BW per day had no effect on any of the safety parameters when administered for one month, nor were there any effects on pups after pregnant female rats were administered with 500 mg/kg BW on days 5 to 17 of pregnancy (Van Rensburg *et al*., in press).

According to a report by the European Agency for the Evaluation of Medicinal Products on toxicity studies (Feb 1999), humic acids extracted from brown coal has no toxic effects at oral dosages as high as 1g/kg BW. It has no terratogenic effects when administered to pregnant rats nor does it have any mutagenic effects according to standard tests for mutagenicity. The LD50 in rats, after oral administration of humic acids, is greater than 11g/kg BW, which was confirmed by Van Rensburg *et al.,* (in press).

Aim

1. To determine the safety profile of potassium humate in volunteers
2. To determine whether potassium humate has anti-allergic properties
3. To determine the effect of potassium humate on basophil activation, cytokine levels and eosinophil migration

Objectives

1. Test the safety of potassium humate in allergic individuals by monitoring haematological, kidney and liver enzyme markers
2. Test the effectivity of potassium humate in individuals suffering from allergic rhinitis
3. Determine the effect of potassium humate on basophil activation, cytokine levels and eosinophil migration *in vivo*

CHAPTER 2
MATERIALS AND METHODS

2.1 Clinical Trial

2.1.1. Primary outcome

To investigate potassium humate vs placebo treatment in patients suffering from hayfever using:

(a) Total symptom score/12h period
(b) Effect Quality of life

2.1.2. Secondary outcomes:

(a) Product safety profile vs placebo
(b) Onset of action
(c) Nasal markers of inflammation
(d) Cutaneous hypersensitivity reaction
(e) Effect on basophil activation
(f) Effect on Th1/Th2 cytokines

2.1.3. Study plan and evaluation of response

In this parallel double-blind placebo controlled phase II study potassium humate (1.8g in divided doses/day) was randomly assigned to atopic patients (n = 40) presenting with acute symptoms of hay fever. The dosage was calculated taking into consideration both the safety and effectivity from studies done on rats (Van Rensburg *et al*., in press).

A minimum of 40 patients will be enrolled in the intention to treat analysis in a single centre in South Africa.

Treatment duration was 30 days, preceded by a one-week (7 days) run-in period.

There were four visits at the investigators' site:

Visit 1: initial visit – screening
Visit 2: randomisation visit within 7 days from visit 1 and after 48 h of moderate to severe symptoms of allergic rhinitis i.e. mean TSS $\geq$ 6
Visit 3: 14 days (+ 3 days) after visit 2 to assess clinical efficacy
Visit 4: 28 days (+ 3) after visit 3 for final assessment

Additional visits will be arranged with patients as dictated by clinical need

2.1.4 Outcome parameters

Total symptom score assessment

Nasal symptoms	Non-nasal symptoms
• Sneezing	Ocular itching (burning scratching etc)
• Running nose	Tearing of eyes
• Nasal itching	Itching of ears/and palate
• Nasal blockage	Ocular redness (conjunctival injection)

To be selected the patient had to have a score of nasal symptoms equal to or greater than 6 ($\geq$ 6 over 12) on both days 48 hours preceding randomization. This is the baseline amount required for participation in this trial as per similar trials studied.

The patient did self-assessment on a diary using the following scoring method:

0 = No symptoms present
1 = Mild symptoms occasionally present but not troublesome
2 = Moderate symptoms, frequently present and annoying
3 = Severe symptoms continuously present and interfering with work or sleep or other daily activity

The safety of potassium humate was determined by means of a full blood count, liver and kidney function tests namely sodium, potassium, creatinine, alkaline phosphatase, gamma GT, urea and urate. Blood samples were sent to Ampath laboratories® (SANAS Accredited Laboratory ISO-IEC 17025 medical testing laboratory # M0066) where the chemistry was done on a Roche Modular P800 and the haematology on a Beckman Coulter LH 750.

The blood and nasal samples that were used to determine the safety of/and the effects of potassium humate on basophil activation, cytokine levels and eosinophil migration, was supplied from the clinical trial. The blood used for the neutrophil adhesion assay was supplied by healthy student volunteers. All the data capture as well as the data analysis was the responsibility of the candidate. The statistical analysis that was done was an analysis of variance (ANOVA) and the data was adjusted for baseline therefore compensating for the inter-subject variability.

2.2. Skin prick testing

Skin prick testing (SPT) is a cheap, rapid and accurate way of identifying the causative allergens in the atopic individual. SPT is uncomplicated and with practise and adherence to a few simple guidelines, it is possible to get highly reproducible results. They are particularly useful for new allergen identification but can also be used in selected cases for the diagnosis in food, drug and insect allergy.

The test is dependant on the introduction of allergen extract into the dermis which then results in an IgE-mediated response, and is then characterized by a wheal and flare reaction. SPT is best performed on the volar or inner aspect of the forearms avoiding the flexures and the wrist areas. A lancet was used to prick the skin through the drop. With the so-called "prick through drop" method it is unnecessary to scratch or lift the skin and no blood should be drawn. The lancet is wiped with dry gauze between each prick, in order to prevent a carry-over of allergens.

Reactions should occur within 10-15 minutes after which the results can be assessed. Positive and negative controls were also included in each series of tests. The negative control solution is the diluent used to preserve the allergen extract. A reaction of 3

mm greater than the negative control is regarded as positive. Grading In conclusion, SPT is safe, simple and cheap, with immediate reproducible results available to the clinician. In conjunction with the case history and clinical findings it remains a valuable diagnostic tool.

2.3. Eosinophil evaluation

2.3.1. Materials

3 different solutions were used to stain the eosinophils collected from the nostrils of the subjects, namely:

Solution 1: Fixative (95% Ethanol)
and
Solution 2: "Rapidiff" Haematology stain 1 - eosin stain (Red) (Microbiology Diagnostic Reagents, Rosettenville, South Africa)
and
Solution 3: "Rapidiff" Haematology stain 2 - methylene blue (Microbiology Diagnostic Reagents)
and
Distilled water for rinsing

2.3.2. Staining method

After optimizing the staining method, it was found that the following method was best suited for distinguishing the number of eosinophils. When the nasal swab was taken it was rolled onto a glass slide and then spread across the slide. The slide was air dried and flooded with the fixative for 5 seconds; the fixative was then gently drained from the slide onto a paper towel. The slide was then flooded with the eosin solution for 40 seconds; the eosin was then gently drained from the slide onto a paper towel. The slide was then flooded with the methylene blue solution for 15 seconds and the methylene blue gently drained from the slide onto a paper towel. The slide was then rinsed by flooding the slide with distilled water for 5 seconds. The slide was then allowed to air dry and examined under oil immersion.

2.3.3. Grading method

A grading scale (see below), as number of eosinophils present per 100 leukocytes, was used to monitor the response to treatment as described in by Berman *et al*., 1991.

Grading used:
1: No cells seen
2: Only occasional eosinophils on smear.
3: Present but scanty and scattered throughout smear.
4: Approximately 15-30 cells
5: Approximately 30-75 cells
6: Approximately > 75 cells

2.4. Basophils

2.4.1. Beckman Coulter Allergenicity Kit (Allergenicity Kit, Cellular Analysis of Allergy)

2.4.1.1. Intended use

The Allergenicity Kit consists of an optimized three-color combination of fluorescent monoclonal antibodies reagent, an activation solution, a positive control for IgE-mediated basophils activation, a stop solution, a lysing and a fixative solution. It is intended for "*In Vitro* Diagnostic Use" for the determination of activated basophils based on accurate basophils gating tool ($CRTH2^{pos}CD203c^{pos}CD3^{neg}$) performed on whole blood specimens.

2.4.1.2. Summary and explanation

The reagents of this kit are designed to identify and differentiate resting and activated basophils by flow cytometry. For this purpose, a 3-color combination is used. It is a mixture of 2-fluorescent murine monoclonal antibodies (CD203c-PE and CD3-PC7) and one-fluorescent rat monoclonal antibody (CRTH2-FITC).

CRTH2

T helper 1 (Th1) and T helper 2 (Th2) lymphocyte subsets are characterized by their cytokine profile production. While Th1 is reported to be involved in cellular immunity, Th2 profile is known to be responsive for humoral immune responses and allergy. Moreover, Thl- and Th2-like cytokine profiles are known as Type I and Type 2 responses (Mosmann *et al*., 1996; Sander *et al*., 1991; Romagnani, 1995; Carter *et al*., 1997).

CRTH2 is a seven-transmembrane molecule known as the Chemoattractant Receptor-homologous molecule that is preferentially expressed on human Th2 and T cytotoxic (Tc2) cells but not on Th1 and Tcl cells (Cosmi *et al*., 2001). Two G protein-coupled receptors, Prostaglandin D receptor (DP), and CRTH2 have been identified as receptors for Prostaglandin D2 (PGD2), but they differ in signaling pathways (Hirai *et al*., 2001, 2002).

PGD2 is the major metabolite of arachidonic acid produced by allergen-activated mast cells and has been implicated in various allergic diseases as a pro-inflammatory lipid mediator (Hirai *et al*., 2002). CRTH2 is also a reliable surface marker selectively associated with circulating T (Th and Tc) cells able to produce IL-4 (as well as IL-5 and lL-l3) but not IFN (Cosmi et al., 2000, 2001).

The monoclonal antibody (mAb) BM 16 precipitates a 55 to 70 kDa protein from cells lysates of CRTH2-transfected JURKAT and from established Th2 clone, (e.g. clone 6L21) corresponding to PGD2 receptor (Nagata *et al*., 1999). Among normal whole blood leucocytes, CRTH2 is highly expressed on basophils and eosinophils, as well as on Th2 and Tc2 cells that are known to be responsive for humoral immune responses and allergy (Nagata *et al*., 1999; Cosmi et al., 2001).

CD203c

Basophils and mast cells are hematopoietic effector cells involved in allergic and inflammatory reactions (Holgate, 2000; Galli, 2000; Johnston *et al*., 1990). Both cell types highly express the high affinity IgE receptor. MAb 97A6 recognizes a surface antigen i.e. 97A6 (CD203c) expressed on human peripheral blood basophils, but not

on other blood cells (Bühring *et a*l., 1999). It also reacts with mature mast cells, and with CD34 bone marrow progenitors of basophils and mast cells. Moreover the 97A6 antigen is up regulated after activation of basophils by anti-IgE antibodies and various allergens (Platz *et al*., 2001).

The basophil activation marker defined by mAb 97A6 is identical to the ecto-nucleotide pyrophosphatase / phosphodiestemse 3 (E-NPP3), a type II transmembrane protein that belongs to a family of ectoenzymes involved in hydrolysis of extracellular nucleotides.

Allergenicity positive control

Basophils express the IgE high affinity receptor. The anti-IgE recognizes the IgE bound to their receptor and consequently induces basophils activation. This reagent provides an IgE-mediated activation positive control.

Allergenicity activation solution

This is an optimized calcium-enriched buffer that allows the *in vitro* activation of basophils from whole blood using EDTA (ethylenediaminetetraacetic acid) as anti-coagulant.

Allergenicity stop solution

This is an optimized EDTA-enriched buffer that permits to stop the *in vitro* activation of basophils.

Allergenicity lysing solution

This reagent is intended for the lysis of red blood cells in the preparation of biological samples for flow cytometric analysis. The major active ingredient is a cyclic amine, which, in contact with carbonic anhydrase present in red blood cells, is transformed into a compound that is highly lytic for these cells.

Allergenicity fixative solution

The allergenicity fixative solution allows whole blood specimen preparation by fixing the cell suspension during erythrolysis. It is also used for fixing the preparation before flow cytometry analysis.

2.4.1.3. Materials

Materials supplied by kit:

The allergenicity kit allows to perform 100 tests and contains the following:

Allergenicity CRTH2-FITC / CD203c-PE / CD3-PC7 – 1 vial (2ml, liquid format) – 20 µl/test.

Allergenicity Positive Control – 1 vial [0.2mg, freeze-dried, in phosphate-buffered saline (PBS) containing 2mg/mL bovine serum albumin (BSA)] – 20µl/test.

Allergenicity Activation Solution – 2 vials. (2x5ml, liquid format containing calcium in buffered solution) – 100µl/test.

Allergenicity Stop Solution – 1 vial (10ml, liquid format containing EDTA and 0.1% sodium azide) – 100µl/test.

Allergenicity Lysing Solution – 3 vials (3x100ml, liquid format containing cyclic amine as major active ingredient) – 2ml/test.

Allergenicity Fixative Solution – 1 vial (10ml, liquid format containing 8% of formic acid).

The reagents of this kit should be used as a set as supplied in the kit and should not be separated. When the tests are performed, care should be taken that all reagents have the same lot number on the vial label.

Materials not supplied by kit:

Deionized water.

PBS buffer

Evacuated blood collection tubes with EDTA anticoagulant

Plastic test tubes (12 x 75mm).

37°C water bath.

Vortex mixer.

Timer.

Calibrated repeater pipette (20µl, 100µl, 2ml) and tips.

Tubes for freezing.

Flow cytometer.

Allergens

2.4.1.4. Principle of test

The cell population of interest is stained with monoclonal antibodies in the presence of the allergen or controls. Erythrocytes are then lysed prior to flow cytometry analysis.

Once excluding T lymphocytes (CD3 positive cells), basophils are analyzed using CRTH2 and CD203c expression. Non-activated and resting basophils are identified as $CRTH2^{pos}CD203c^{dim}CD3^{neg}$, whereas *in vitro* activated basophils are identified as $CRTH2^{pos}CD203c^{bright}CD3^{neg}$.

For each blood specimen, a minimum of three analysis are performed:

♦ Tube # "Neg" = Negative Control Tube

♦ Tube # "Pos" = Positive Control Tube

♦ Tubes "Test" (it is recommended to use different dilutions of the allergen)

For each individual the allergen [Laboratory Specialities (PTY) LTD, Johannesburg, South Africa] used was the one that the participant was most allergic to as detected during the skin prick test at the screening part of the trial.

The red blood cells in each sample are lysed with a "Fix-and-Lyse" mixture and the remaining cells analyzed by flow cytometry.

The flow cytometer analyzes light diffusion and the fluorescence of cells. It makes possible the delimitation of the population of interest within the electronic window defined on a histogram, which correlates the orthogonal diffusion of light (Side Scatter or SS) and the diffusion of narrow-angle light (Forward Scatter or FS). Histograms combining two of the different parameters available on the cytometer are used as supports in the gating stage for the application.

The fluorescence of the delimited cells is analyzed in order to distinguish the positively stained events from the unstained ones.

2.4.1.5. Method

Preparation of reagents

All reagents were brought to room temperature (18 – 25°C) prior to experiment.

The kit consisted of ready-for-use reagents:

- CRTH2-FITC / CD203c-PE / CD3-PC7.

- Allergenicity Activation Solution.

- Allergenicity Stop Solution.

No reconstitution was necessary and reagents were used directly from the vial.

Allergenicity positive control

Freeze-dried positive control was reconstituted by addition of 1ml of 0.22µm filtered deionised water in order to obtain the concentration of 0.2mg/ml. A sufficient amount of the positive control was prepared for the total number of sample preparations.

"Fix-and-Lyse" preparation

Allergenicity Lysing Solution (3x100ml). Reagent was used directly from the vial.

Allergenicity Fixative Solution (1x10ml). Reagent was used directly from the vial.

A "Fix-and-Lyse" mixture using Allergenicity Lysing Solution and Allergenicity Fixative Solution was prepared daily for the total number of sample preparation.

A sufficient amount of the "Fix-and-Lyse" mixture was prepared for the total number of sample preparation.

PBS 0.1% formaldehyde preparation

PBS 0.1% formaldehyde was prepared by mixing 1ml of PBS with 12.5µl (example for two tubes) of allergenicity fixative solution. Prepare a sufficient amount of PBS 0.1% formaldehyde for the total number of sample preparation.

Specimen Requirements

Peripheral blood samples were collected aseptically into a sterile evacuated blood collection tube with EDTA as anticoagulant. EDTA-collected specimens were stored at room temperature (18 – 25°C) until processing.

EDTA-collected were less than 24 hours old for processing.

Fresh specimens were used.

All specimens were processed with the kit reagents.

The specimen was kept at room temperature (18-25°C) and not shaken. The specimen was homogenized by gentle agitation prior to pipetting. Samples were processed within 24 hours of taking them.

Procedure for instrument adjustment

The flow cytometer is equipped to detect forward scatter, side scatter and three fluorescence channels allowing the analysis of FITC-, PE-, and PC7- conjugated

antibodies (respectively 504 – 541 / 568 – 590 / 750 – 810nm maximal peak emission).

Ensured that the flow cytometer is properly aligned and standardized for fluorescence intensity (according to the manufacturer recommendations).

Ensured that the flow cytometer is properly adjusted for fluorescence compensation.

Procedure for specimen processing

The following procedure was optimized for whole blood specimen collected with EDTA as anticoagulant.

All reagents were brought to room temperature (18 – 25°C) prior to experiment.

The right range of allergens dilutions to be used for the experiment.

For each experiment, the appropriate numbers of tubes were labelled:

• Tube # "Neg" = Negative Control Tube

• Tube # "Pos" = Positive Control Tube

• Tubes "Test" (different dilutions of the appropriate allergen)

Added 20µl of PBS on tube # "Neg", 20µl of allergenicity positive control on tube # "Pos", 2µl of tested allergen on tubes "Test". Then 20µl of CRTH2-FITC / CD203c-PE / CD3-PC7 reagent was added to each tube. Then 100µl of activation solution was added to each tube. 100µl of whole blood was then pipetted into each tube.

The tubes were then gently vortexed and incubated for 15 minutes at 37°C in water bath, protected from light. Then 100µl of stop solution was added to each tube, and gently vortexed for 5 seconds. Then 2ml of "Fix-and-Lyse" mixture was added to each tube and vortexed. The tubes were then incubated at room temperature (18 – 25°C) for 10 minutes, protected from light.

The tubes were then centrifuged for 5 minutes at 200 g, and the supernatant aspirated. 3ml of PBS was added to each tube. The tubes were the centrifuged for 5 minutes at

200 g, and the supernatant aspirated. The cell pellet was resuspended in 0.5ml of PBS 0.1 % formaldehyde. It was then acquired on the flow cytometer.

2.5. Cytokine assay

2.5.1. FlowCytomix human Th1/Th2 10plex (hIFN-γ, hIL-1β, hIL-2, hIL-4, hIL-5, hIL-6, hIL-8, hIL-10, hTNF-α, hTNF-β) kit.

2.5.1.1. Intended use

Multiplex Fluorescent Bead Immunoassay (FBI) for quantitative detection by flow Cytometer of human Interferon-γ, Interleukin-1β, Interleukin-2, Interleukin-4, Interleukin-5, Interleukin-6, Interleukin-8, Interleukin-10, tumour necrosis factor (TNF) α and β in cell culture supernatants, serum, plasma, whole blood, or other body fluids. It is for research use only. Not for use in diagnostic or therapeutic procedures.

2.5.1.2. Summary

The term Th1 cytokines (referred to also as Type-1 cytokines) and Th2 cytokines (referred to also as Type-2 cytokines) refers to the patterns of cytokines secreted by two different subpopulations of murine CD4 (+) T cells that determine the outcome of an antigenic response toward humoral or cell-mediated immunity.

Numerous cells other than T-cells expressing CD4 have been shown to be capable of producing Th1 and Th2 cytokines. These cells include CD8 (+) T-cells, monocytes, natural killer cells, B-cells, eosinophils, mast cells, basophils, and other cells.

Type-1 cytokines include IL-2, IFN-γ, IL-12 and TNF-β, while Type-2 cytokines include IL-4, IL-5, IL-6, IL-10 and IL-13. Type-1 helper cells (Th1), but not type-2 helper cells (Th2), secrete IL-2, IFN- γ and TNF- β, whereas Th2 cells, but not Th1 cells, express IL-4, IL-5, IL-6 and IL-10 (Linden *et al*., 1995; Durham *et al*., 1996; Pawankar *et al*., 1996; Klimek *et al*., 1999; Benson *et al*., 2000).

The molecular mechanisms underlying the evolution of these two different cell types from common precursors are still not completely known. Studies with transgenic mice carrying null mutations of the IL-4 gene have shown that IL-4 plays an important role in the establishment of a functional Th2 immune response. The different patterns of cytokine secretion correspond with different functions as immune effectors.

Th1 cells promote cell-mediated effector responses. Th2 cells are mainly helper cells that influence B-cell development and augment humoral responses such as the secretion of antibodies, predominantly of IgE, by B-cells. Both types of Th cells influence each other by the cytokines they secrete; IFN-γ, for example, can down regulate Th2 clones while Th2 cytokines, such as IL-10 can suppress Th1 functions. IFN-γ has been shown also to inhibit the proliferation of murine Th2 cells but not that of Th1 helper T-lymphocyte clones. It thus appears that these functional subsets are mutually antagonistic such that the decision of which subset predominates within an infection may also determine its outcome.

2.5.1.3. Materials

The kit, except for the following, supplied all materials:

A flow cytometer (FC 500) equipped with one laser (488nm or 532nm) capable of detecting and distinguishing fluorescence emissions at 575nm and far red (685 - 690 nm)

Sample acquisition tubes for a flow cytometer

Aluminium foil

5ml and 10ml graduated pipettes

10µl to 1,000µl adjustable single channel micropipettes with disposable tips

20µl to 300µl adjustable multichannel micropipettes with disposable tips

Multichannel micropipette reservoir

Beakers, flasks, cylinders necessary for preparation of reagents

Glass-distilled or deionised water

Vortex mixer

2.5.1.4. Principle of test

Microspheres are coated with antibodies specifically reacting with each of the analytes to be detected in the multiplex system. The beads can be differentiated by their sizes and by their distinct spectral addresses. A mixture of coated beads for each analyte to be measured is incubated with the samples or standard mixture. The analytes present in the sample bind to the antibodies linked to the fluorescent beads.

A biotin-conjugated second antibody mixture is added, the specific antibodies bind to the analytes captured by the first antibodies. Phycoerythrin is added, binds to the biotin conjugate and emits fluorescent signals.

2.5.1.5. Method

Plasma was used in the assay. Plasma was removed from the clot, as soon as possible after clotting and separation.

Fat causes agglutination of the beads. Centrifugation of lipemic samples (about 16.000 rcf for 5 min) before analysis was done.

Clinical samples were kept between 2° to 8°C and separated rapidly before storing at -20°C to avoid loss of bioactivity.

All reagents were brought to room temperature and vortexed well before usage.

Preparation of assay buffer

The contents of the bottle were mixed well. 50ml assay buffer concentrate was added to 450ml distilled water and mixed gently to avoiding foaming and stored at 2° to 8°C.

Preparation of standard

The number of analytes was determined. The vials were centrifuged for a few seconds in a micro centrifuge before opening to collect lyophilized standard at the bottom. The lyophilized standard was reconstituted by adding distilled water according to the label on the standard vial. The vial was swirled thoroughly to ensure quantitative solubilization of contents. Waited for ten minutes before pipetting the standard. The final volume of the standard mix was 200µl.

10µl of each reconstituted standard were added to a vial labelled Standard 1. The vial was then filled up to a final volume of 200µl with Assay Buffer.

Creating standard mixture

A 1:20 dilution of each reconstituted standard was made.

The vials were centrifuged for a few seconds in a micro centrifuge before pipetting reconstituted standard.

Serial dilutions for the preparation of the standard mixture

100µl assay buffer (1x) was added to 6 tubes labelled standard 2 to 7. 50µl of standard 1 was then transferred to tube labelled 2, the contents of tube 2 were then mixed and 50µl was transferred to tube labelled 3. Repeated the procedure creating a row of 7 standard dilutions. Any solubilized or prediluted standard left after usage was discarded.

Preparation of bead mixture

The bead mixture was prepared according to the following calculation:

Per well 25µl of the bead mixture was required. Considered that 17 wells were needed for 2 standard curves and 2 blanks plus an additional standard 1 for setup. Then final volume (Vfin) of the bead mixture needed was calculated.

Vfin = number of wells x 25µl. Rounded up for pipetting reservoir. The individual bead vials were vortexed for 5 seconds and pipetted 1/20 of final volume (Vfin) of each bead set to a vial labelled "Bead Mix". Filled up to the final volume (Vfin) with Reagent Dilution Buffer (RDB).

Preparation of biotin-conjugate

The biotin-conjugate mixture was prepared according to the following calculation:

Per well 50µl of the biotin-conjugate mixture was required. Considered that 17 wells were needed for 2 standard curves and 2 blanks plus an additional standard 1 for setup. The final volume (Vfin) of the biotin-conjugate mixture needed was calculated. Vfin = number of wells x 50µl. Rounded up for pipetting reservoir.

Individual bead vials were vortexed for 5 seconds and pipetted 1/20 of final volume (Vfin) of each Biotin-conjugate to a vial labelled "Biotin-conjugate Mix". Filled up to the final volume (Vfin) with Reagent Dilution Buffer (RDB).

Preparation of streptavidin-PE

A dilution of the concentrated streptavidin-PE solution in Assay Buffer (1x) was made.

Test procedure using tubes

In this case incubations were performed in tubes. Centrifugation steps were necessary for test performance. The number of cytometer tubes required was determined to test the desired number of samples plus appropriate number of tubes needed for running blanks and standards. 25µl of standard mixture dilutions 1 to 7 was added to designated tubes. 25µl of Assay Buffer was added to the blank tubes. 25µl of standard mixture dilution 1-17 was added, which is designated for the flow cytometer setup. 25µl of sample was added to the designated sample tubes. 25µl of bead mixture was added to all the tubes, including the blank tubes. 50µl of biotin-conjugate mixture was added to all the tubes, including the blank tubes. The contents of each tube was mixed and incubated at room temperature (18° to 25°C) for 2 hours, protected from light with aluminium foil. The streptavidin-PE was then prepared.

1ml of assay buffer was added to all tubes and spun down at 200 x g for 5 minutes. The supernatant was then carefully discarded from each tube, leaving 100µl of liquid in each tube. This step was then repeated. 50µl of streptavidin-PE was then added to all the tubes including the blank tubes. The contents of each tube were mixed well and incubated at room temperature (18° to 25°C) for 1 hour, protected from light with aluminium foil.

1ml of assay buffer was added to all tubes and spun down at 200 x g for 5 minutes. The supernatant was then carefully discarded from each tube, leaving 100µl of liquid in each tube. This step was then repeated. 500µl of the assay buffer was then added to each tube. It was then acquired on the flow cytometer.

2.6. Neutrophil adhesion assay

This assay was done as described by Håkanson *et al*., 1994 and on blood drawn from healthy student volunteers.

2.6.1. Materials

Venous blood was mixed with 5 U preservative-free heparin per milliliter blood.

Willing healthy donors were used and notes were made on the amount of blood taken from individuals so that they were not used too frequently.

Instrumentation:

A temperature-controlled centrifuge was used at 4^0C for the centrifugation steps. A microscope was used to enumerate the cells and a vortex was used to resuspend the cells.

Preparations of reagents:

The following reagents were prepared from the isolation of neutrophils:

Heparin

90mg of Heparin (Sigma Diagnostics, St Louis, MO, USA) was diluted in 30ml of distilled water. It was then mixed, filtered and sterilized. An amount of 0.1ml of heparin was used for every milliliter of blood. The solution was stored at 4°C.

Histopaque

Histopaque (Sigma) was used as is and was stored at 4°C.

Ammonium chloride

8.3g Ammonium chloride (Merck, Darmstadt, Germany), 1g $NaHCO_3$ (Merck) and 74mg EDTA (Sigma) were dissolved in 1L of distilled water and stored at 4°C.

Phosphate buffered saline

0.923mg BBL™ FTA Haemaglutination Buffer (BBL Microbiology Systems, Becton Dickenson and Company, USA)
of was solubilised in 100ml distilled water and checked to ensure that it was at a pH of 7.4.

Hanks Balanced Salt Solution (HBSS)

HBSS (Highveld Biologicals (Pty) Ltd., Johannesburg, SA) was made up according to the manufacturer's instructions.

Leucocyte counting fluid

0.1ml of a 0.1 % (100mg/100 ml) gentian violet solution (Merck) was added to 2ml glacial acetic acid (Saarchem-Holpro Analytics Ltd). Then 100ml of distilled water was added and the solution was stored at 4°C.

The following reagents were used for the neutrophil adhesion assay:

Assay buffer:

8.1816 g NaCl (Labchem Ltd)

0.3728 g KCl (Saarchem-Holpro Analytics Ltd)

0.2033 g MgCl (Saarchem-Holpro Analytics Ltd)

0.1110 g/L CaCl (BDH Ltd Poole England)

0.50445 g/L Glucose (BDH Ltd Poole Engeland)

2.383 g/L HEPES (Sigma)

30 mg human serum albumin (Sigma)

62.9 mg/L $MnCl_2$ (Merck, Darmstadt, Germany)

All the above components were solubilised in 1L of distilled water and the pH adjusted to 7.4.

Phorbol myristate acetate (PMA) (stimulant)

20µl PMA (Sigma) stock solution (1mg/ml DMSO) was added to 1ml distilled water. This solution was diluted by adding 100µl of the original solution to 1.9ml distilled water. It was then further diluted by adding 1ml of the new solution to 3ml of distilled water.

O-phenylenediamine Solution (OPD)

A. Citrate phosphate buffer:

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

Na_2HPO_4 (MW 142.0) 9.5 14g/L (Saarchem-Holpro Analytics (Pty) Ltd)

Above mentioned buffer was prepared as follows:

The reagents were dissolved separately in 400ml of distilled water. Small amounts of Na_2HPO_4 were added to the citric acid while stirring frequently. The pH of the solution was checked frequently, when everything is mixed together, the pH should be almost 5. The solution was then made up to 1L and a pH of 5 using distilled water.

B. TRITON X-100

1 g/L of TRITON X-100 (0.1%) (Sigma) was used.

C. O-phenylenediamine (OPD)

1 g/L of OPD (Sigma) was used.

D. H_2O_2

(Stock solution = 10mol)

400µl/L of H_2O_2 (4µmol/L) (Sigma) was used.

Solutions A, B, C and D were mixed together. This is called the substrate solution.

H_2S0_4 (stop solution) (1mol/L) (Saarchem-Holpro Analytics (Pty) Ltd., Krugersdorp, SA)

28ml in 1L distilled water.

2.6.2. Method (Böyum, 1968)

500µl heparin was added to a urine bottle or blood collection bag. The required volume of blood and heparin were then mixed well together. 15 ml of Histopaque was then added to a 50 ml graded, plastic tube (Ficoll tube). The blood was then carefully

layered onto the Histopaque and the tube filled to the top. The tubes were then centrifuged for 25 minutes at 1800 rpm in a centrifuge set to room temperature.

The top layers containing plasma, platelets, monocytes, lymphocytes and histopaque were removed, using a plastic pipette and discarded into a plastic container, together with a 5 % hypochlorite solution. The remaining layer is the granulocyte layer which contains the required neutrophils. The granulocyte layer and ± 3 mm from the top of the red blood cell suspension was removed and pipetted into a clean 50ml tube and filled up with ice cold 0.84 % ammonium chloride (NH_4CI). The contents were mixed well and left to stand for 10 minutes in ice. The red blood cells were lysed during this step.

The tube was then centrifuged for 10 minutes at 1200 rpm. The resultant supernatant fluid was discarded and the pellet kept. If the pellet still contained red blood cells (reddish colour), the ammonium chloride and centrifuge step were repeated. The white pellet that was kept contains granulocytes of which neutrophils are the most abundant.

The pellet was then vortexed and the tube filled up with phosphate buffered saline. In the case of several tubes, they were all pooled at this stage. This step was included to wash off any remaining ammonium chloride. The tube was centrifuged for 10 minutes at 1100 rpm. The resultant supernatant fluid was discarded. The tube was then vortexed and the cells resuspended in cold PBS and then diluted with PBS to a concentration of 2.5 X 10^6 cells/ml using a haemocytometer. Cells were kept on ice until used.

Polymorphonuclear leucocytes adhesion, experimental procedure

Round-bottomed 96-well tissue culture plates (sterile) were used.

The following was added to the wells:

DMEM	100µl
BHK 238-1/331-7 cells	100µl

The cells were then incubated for 24 hours at 37^0C in a CO_2 incubator

The fibroblast monolayers were washed in the 96 well microtiter plate three times with assay buffer. To each well 140µl assay buffer was added. Then 20µl of the potassium humate (made up in DMEM) was added (final concentration of potassium humate = 100µg/ml). The cells were incubated for 15min at 37^0C in a CO_2 incubator. Then 20µl of a PMNL suspension (2.5 x 10^6/ml) (PMNL: FIBRO = 5:1 for optimal adhesion) was added to each well after which 20µl PMA solution (stimulant) or 20µl distilled water (resting) was added.

Plates were incubated for 1 hour at 37^0C. After incubation non-adherent PMNL were removed from the wells and the plates were washed three times with assay buffer. 100µl OPD substrate solution was added and incubated for 5 min. 100µl H_2S0_4 was added to each well to stop the reaction. Absorbance was read at 492 nm.

The percentage of adherent granulocytes of the total amount (2.5 x 10^6/ml) that initially had been added to the wells were calculated from a standard curve, consisting of serial dilutions (1 to 100%) of the initial amount of PMNL (using simple regression analysis) as follow:

5 dilutions were made from the initial amount (2.5 x 10^6/ml) PMNL in assay buffer. 140µl assay buffer was added to 12 wells of a 96-well microtiter plate. 20µl of the diluted suspensions of PMNL, 2.5 x 10^6, 1.25 x 10^6, 0.6x 10^6, 0.3 x 10^6, 0.15 x 10^6, and 0.07 x 10^6 was added in duplicate. Then 40µl of distilled water was added. The plates were then incubated for 1 hour at 37ºC in a $C0_2$ incubator.

The plate was then centrifuged for 8min/1500rpm. The supernatant was carefully removed with a pipette without disturbing the pellet. 100µl OPD was added to the substrate solution and incubated for 5 min. Then 100µl H_2SO_4 (1mol/L) was added to each well to stop the reaction. Absorbance was read at 492nm.

CHAPTER 3
RESULTS

3.1. Safety testing

The clinical safety of potassium humate was determined by means of a full blood count, liver and kidney function tests namely sodium, potassium, creatinine, alkaline phosphatase, gamma GT, urea and urate. Blood samples were taken from the patients before and after the trial and were sent to Ampath laboratories® for testing.

After analysis of the pathology results it was observed by means of an ANOVA that all the parameters were within the safe range and there was no significant differences with respect to the placebo vs the treated group, thus proving the potassium humate to be safe to use at this dose (Table 1, Figures 1 and 2).

Table 1: Pathology results for the full blood count (HGB, RBC, HCT, MCV, MCH, MCHC, RDW, WBC, NE, LY, MO, EO, BA, PLT), liver (alkaline phosphatase, gamma GT) and kidney (sodium, potassium, creatinine, urea and urate) function tests of the patients obtained before and after the study of the treated vs placebo group.

	Treated				Placebo			
	Before		After		Before		After	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
HGB (g/dL)	14.14	1.45	14.4	1.10	14.10	1.18	14.08	1.38
RBC (10∃12/L)	4.69	0.50	4.80	0.37	4.76	0.33	4.76	0.37
HCT (%)	41.46	4.10	42.29	2.76	41.21	4.22	41.38	3.88
MCV (fL)	88.58	2.86	88.28	2.98	87.07	4.60	86.99	4.98
MCH (pg)	30.22	1.30	30.09	1.35	29.55	1.75	29.64	1.97
MCHC (g/dL)	34.09	0.75	34.08	0.71	33.93	0.73	34.05	0.62
RDW (%)	12.97	0.74	13.15	0.82	13.31	1.03	13.27	0.95
WBC (10∃9/L)	8.11	2.85	7.36	2.30	7.27	2.14	7.03	1.62
NE (10∃9/L)	4.92	2.60	4.42	2.06	4.37	1.97	4.02	1.59
LY (10∃9/L)	2.43	0.50	2.20	0.50	2.15	0.42	3.54	5.48
MO (10∃9/L)	0.52	0.25	0.54	0.17	0.46	0.15	0.82	1.72
EO (10∃9/L)	0.20	0.15	0.23	0.14	0.24	0.20	0.22	0.13
BA (10∃9/L)	0.02	0.03	0.01	0.02	0.02	0.04	0.08	0.17
PLT (10∃9/L)	289.35	74.35	299.05	77.74	310.50	66.08	320.900	60.42
NA (mmol/L)	137.90	1.80	138.60	1.96	137.90	1.95	138.50	1.85
K (mmol/L)	4.31	0.42	24.51	90.03	4.52	0.49	4.37	0.42
CRE (µmol/L)	72.15	18.51	73.95	12.93	75.35	12.98	75.45	13.17
ALP (U/L 37°C)	56.15	11.65	57.00	11.23	58.16	12.97	58.40	14.63
GGT (U/L)	15.10	9.52	17.55	8.88	15.00	6.90	15.35	6.95
URIC (mmol/L)	0.26	0.08	0.27	0.06	0.275	0.08	0.28	0.08
UREA (mmol/L)	3.74	1.38	3.90	1.05	4.18	0.91	4.06	1.16

HGB: Haemoglobin, RBC: Red blood cells, HCT: Haematocrit, MCV: Mean cell volume: MCH: Mean cell haemoglobin, MCHC: Mean cell haemoglobin concentration, RDW: Red cell distribution width, WBC: White blood cell, NE: Neutrophils, LY: Lymphocytes, MO: Monocytes, EO: Eosinophils, BA: Basophils, PLT: Platelets, NA: Sodium, K: Potassium, CRE: Creatinine, ALP: Alkaline phosphatase, GGT: Gamma-glutamine transaminase.

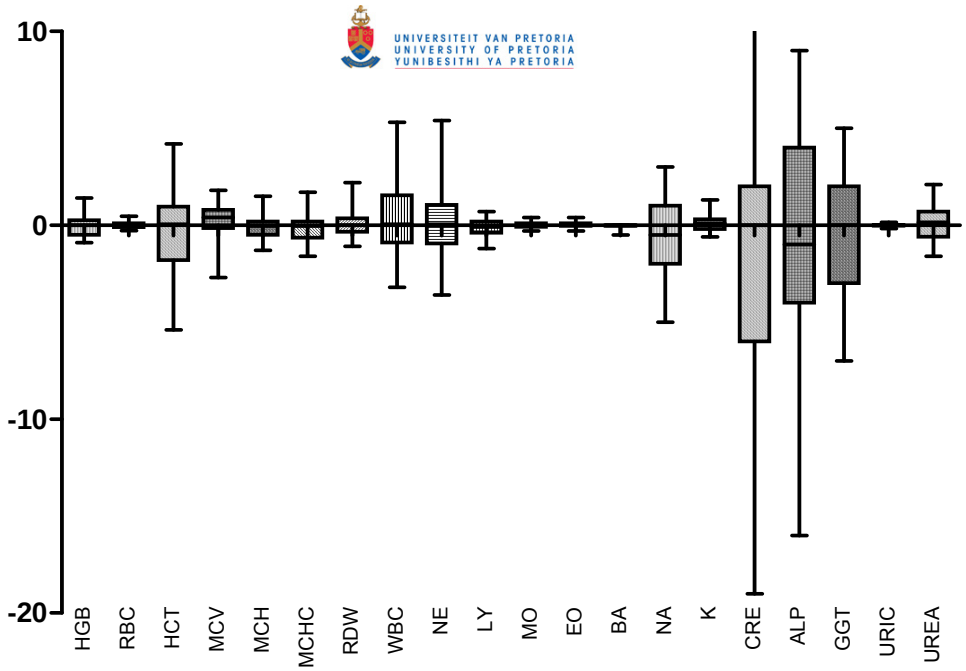

Figure 1: Pathology results for the full blood count (HGB, RBC, HCT, MCV, MCH, MCHC, RDW, WBC, NE, LY, MO, EO, BA, PLT), liver (alkaline phosphatase, gamma GT) and kidney (sodium, potassium, creatinine, urea and urate) function tests that were done on the placebo group.

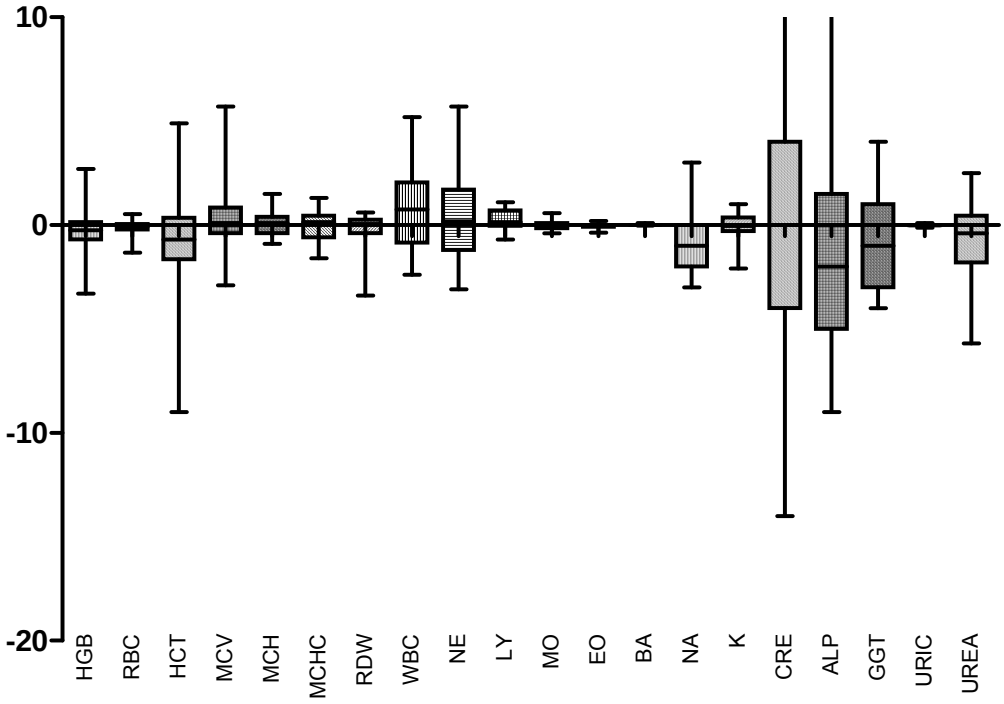

Figure 2: Pathology results for the full blood count (HGB, RBC, HCT, MCV, MCH, MCHC, RDW, WBC, NE, LY, MO, EO, BA, PLT), liver (alkaline phosphatase, gamma GT) and kidney (sodium, potassium, creatinine, urea and urate) function tests that were done on the potassium humate treated group.

3.2. Total Symptom Score

When analyzing the data obtained from comparing the scores for the various times using an ANOVA and adjusting for baseline no significant differences were observed when comparing the placebo group with that of the treated group (Table 2).

Table 2: Total symptom scores before and after the study of the treated vs placebo groups.

	First 48 Hours		1st Mid 48 Hours		2nd Mid 48 Hours		3rd Mid 48 Hours		Last 48 Hours	
	Morning	**Evening**	**Morning**	**Evening**	**Morning**	**Evening**	**Morning**	**Evening**	**Morning**	**Evening**
Treated	**30.8**	**33.7**	**29.8**	**31.1**	**26.2**	**26.7**	**26.7**	**26.8**	**26.4**	**28.6**
Placebo	**30.1**	**34.5**	**28.5**	**31.3**	**24.7**	**28.3**	**22.6**	**25.7**	**22.0**	**24.2**

Morning: Average score for 2 mornings.

Evening: Average score for 2 evenings.

First 48 Hours: Average scores of first 2 days of the trial.

1st Mid 48 Hours: Average scores of days 7 and 8 of the trial.

2nd Mid 48 Hours: Average scores of days 14 and 15 of the trial.

3rd Mid 48 Hours: Average scores of days 21 and 22 of the trial.

Last 48 Hours: Average scores of last 2 days of the trial.

3.3. Skin Prick Test

For each participant the skin prick test was done as part of the screening process and from the result the allergen the participant was most allergic to was then selected along with the positive histamine to be repeated at the end of the trial.

With statistical analysis, using an ANOVA and adjusting for baseline, a significant difference was observed with a $p < 0.05$ when comparing the before and after results of the treated vs placebo groups respectively (Table 3).

Table 3: Measurements of wheal (diameter in mm) before and after the study of the treated vs placebo groups with respect to their skin prick tests.

	Diameter of wheal (mm)			
	Before		After	
	Mean	SD	Mean	SD
Treated	8.69	4.71	*6.26	2.79
Placebo	10.66	3.85	9.67	3.91

* Indicating statistical significance ($p < 0.05$)

3.4. Eosinophils count

A nasal eosinophilia is regarded as a hallmark of nasal allergy (Gleich *et al*., 1994). Eosinophils are not normally seen on a stained smear of nasal secretions. The increased presence of eosinophils may involve a balance of recruitment including chemotaxis of mature cells, proliferation of their progenitors and elimination of tissue eosinophils (Gibson *et al*., 1990). A grading scale may be used to monitor the response to treatment.

With statistical analysis, using an ANOVA and adjusting for baseline, a significant difference was observed in the results expressed as an arbitrary number (between 1-6) corresponding to the degree of eosinophils present on the smear when comparing the before and after results of the treated vs placebo groups respectively (Table 4).

Table 4: Degree of eosinophil presence (using the grading system) in smears obtained from the treated vs placebo groups.

	Degree of eosinophil presence			
	Before		After	
	Mean	SD	Mean	SD
Treated	2.63	1.12	*1.84	0.60
Placebo	2.80	0.83	2.95	0.76

* Indicating statistical significance ($p < 0.05$)

3.5. Neutrophils adhesion assay

This assay was run in order to determine whether the potassium humate has a general anti-allergic action or whether the action has a more specific anti-allergic action. The neutrophil adhesion assay measures the amount of neutrophils that adhered to the BHK 238-1 and 331-7 cell lines respectively. The BHK 331-7 contains ICAM-1 and is therefore more specific for the binding of neutrophils to ICAM-1. Both stimulated and resting cells were measured. Adherent neutrophils were quantitated by analyzing MPO release. Each assay was run in triplicate and then repeated three times, the results were then pooled and an average and standard deviation (SD) was then calculated from these values (Tables 5 and 6) and diagrams drawn (Figures 3 and 4).

As observed from the results there were no significant differences between the stimulated and resting cells as well as no significant difference with relation to the range of increasing concentrations of cells that were treated with potassium humate, thus suggesting that potassium humate has a more specific anti-allergic action. This was observed for both the BHK 238-1 cell line as well as the BHK 331-7 cell line respectively (Tables 5 and 6, Figures 3 and 4).

Table 5: Effects of potassium humate on adhesion of resting vs stimulated neutrophils on adhesion to the BHK 238-1 cell line when treated with increasing concentrations of potassium humate.

	Adhesion average as % of control						
Potassium humate (µg/ml)	**5**	**10**	**20**	**40**	**60**	**80**	**100**
Resting							
Average	**99.55**	**100.10**	**105.94**	**104.19**	**113.18**	**118.67**	**120.50**
SD	**7.97**	**5.24**	**8.39**	**7.67**	**13.95**	**18.17**	**23.31**
Stimulated							
Average	**100.17**	**99.62**	**100.69**	**100.25**	**100.65**	**101.00**	**101.05**
SD	**4.86**	**3.91**	**2.52**	**3.96**	**2.65**	**2.25**	**0.95**

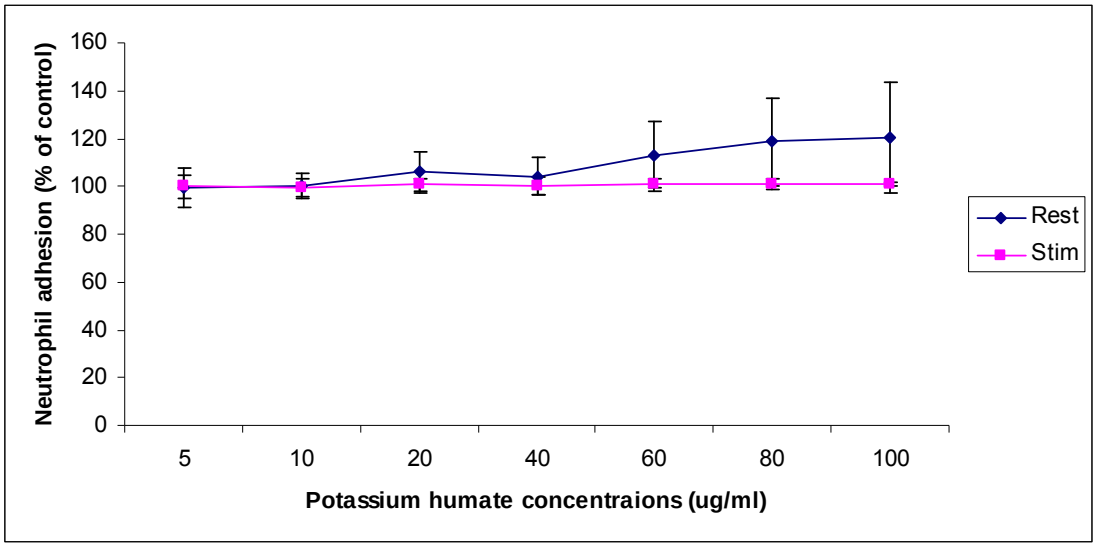

Figure 3: Effects of potassium humate on adhesion of resting vs stimulated neutrophils on adhesion to the BHK 238-1 cell line when treated with increasing concentrations of potassium humate.

Table 6: Effects of potassium humate on adhesion of resting vs stimulated neutrophils on adhesion to the BHK 331-7 cell line when treated with increasing concentrations of potassium humate.

	Adhesion average as % of control						
Potassium humate (µg/ml)	**5**	**10**	**20**	**40**	**60**	**80**	**100**
Resting							
Average	**100.63**	**97.04**	**101.13**	**108.05**	**115.49**	**121.69**	**123.97**
SD	**6.89**	**3.70**	**4.79**	**10.88**	**12.86**	**21.57**	**21.15**
Stimulated							
Average	**95.08**	**95.37**	**97.08**	**99.16**	**99.85**	**98.91**	**101.81**
SD	**4.75**	**8.64**	**5.2**	**6.98**	**3.74**	**4.16**	**3.00**

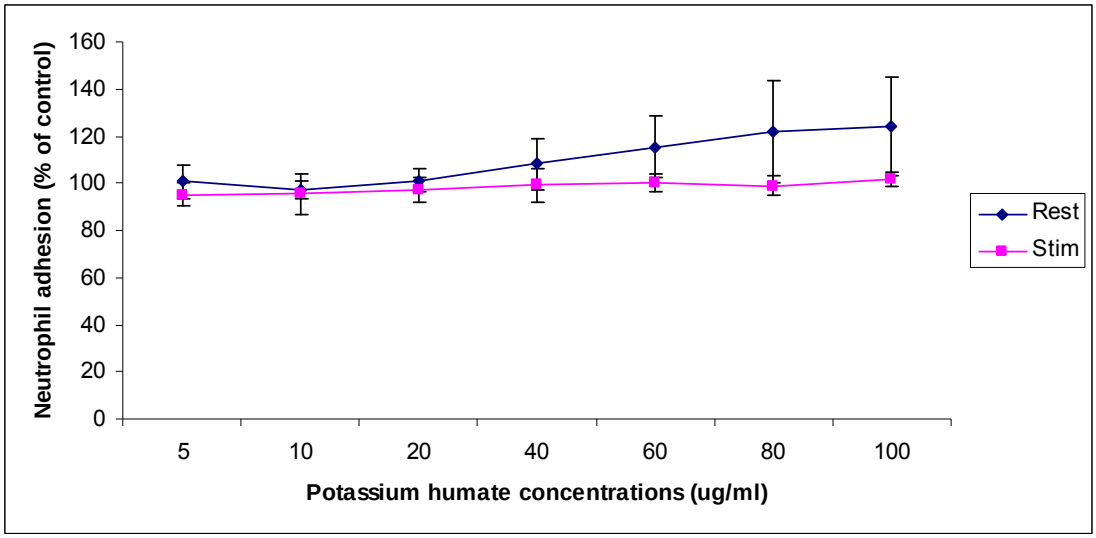

Figure 4: Effects of potassium humate on adhesion of resting vs stimulated neutrophils on adhesion to the BHK 331-7 cell line when treated with increasing concentrations of potassium humate.

3.6. Basophils

The cell population of interest was stained with monoclonal antibodies in the presence of the allergen or controls. The fluorescence of the delimited cells is analyzed in order to distinguish the positively-stained events from the unstained ones. This procedure is carried out twice per patient eg. Before (Visit 2) and after (Visit4) and the results are expressed as a percentage of activated basophils.

When analyzing the data using the difference between the positive and the test and then also the difference between the negative and the test using an ANOVA and adjusting for baseline no significant differences were observed when comparing the placebo group with that of the treated group with respect to their before and after results (Table 7).

Table 7: Placebo vs treated groups with respect to the difference in percentage of activated basophils before and after treatment.

	Visit 2			Visit 4		
	% Activated Basophils			% Activated Basophils		
Placebo	**Positive**	**Negative**	**Test**	**Positive**	**Negative**	**Test**
Average	**42.05**	**8.05**	**50.71**	**43.79**	**8.32**	**49.05**
SD	**27.33**	**11.22**	**31.89**	**24.57**	**15.11**	**29.26**
Treated						
Average	**40.75**	**5.06**	**41.22**	**38.87**	**8.63**	**47.53**
SD	**25.2**	**7.36**	**19.83**	**25.94**	**17.42**	**26.51**

3.7. Cytokines levels

Microspheres are coated with antibodies specifically reacting with each of the analytes to be detected in the multiplex system. The beads can be differentiated by their sizes and by their distinct spectral addresses.

During the clinical trial serum from before and after the trial for each participant was frozen away in order to analyse the cytokines at the same time using the FBI.

When analysing the data for both the pro and anti inflammatory cytokines using an ANOVA and adjusting for baseline, no significant differences were observed when comparing the treated vs the placebo group (Tables 8 and 9).

Th1 Cytokines

Table 8: Average means and standard deviations of the Th1 cytokines before and after the trial respectively.

	Cytokine levels (pg/ml)											
Cytokines	**IFN-G**		**IL-1B**		**IL-2**		**IL-8**		**TNF-A**		**TNF-B**	
	Before	**After**	**Before**	**After**	**Before**	**After**	**Before**	**After**	**Before**	**After**	**Before**	**After**
Placebo												
Average	**24.66**	**28.59**	**21.93**	**29.77**	**41.56**	**46.09**	**96.66**	**126.23**	**31.38**	**58.89**	**51.15**	**62.59**
SD	**9.45**	**19.62**	**9.88**	**23.5**	**18.38**	**27.91**	**51.67**	**89.13**	**21.95**	**77.33**	**43.55**	**55.34**
Treated												
Average	**30.22**	**40.17**	**57.69**	**52.65**	**30.23**	**26.28**	**30.23**	**26.28**	**31.92**	**43.34**	**74.89**	**72.06**
SD	**24.37**	**49.45**	**75.94**	**55.65**	**28.47**	**26.62**	**28.47**	**26.62**	**19.84**	**39.84**	**70.67**	**56.37**

Th2 Cytokines

Table 9: Average means and standard deviations of the Th2 cytokines before and after the trial respectively.

	Cytokine levels (pg/ml)							
Cytokines	**IL-10**		**IL-6**		**IL-4**		**IL-5**	
	Before	**After**	**Before**	**After**	**Before**	**After**	**Before**	**After**
Placebo								
Average	**19.66**	**24.15**	**9.25**	**11.84**	**450.94**	**455.98**	**59.79**	**110.97**
SD	**14.03**	**20.91**	**5.86**	**9.1**	**1847.11**	**1397.49**	**54.04**	**176.98**
Treated								
Average	**21.72**	**265.67**	**474.73**	**556.63**	**119.91**	**110.94**	**19.05**	**14.58**
SD	**13.56**	**992.31**	**1784.16**	**2081.55**	**78.65**	**110.98**	**30.76**	**16.57**

CHAPTER 4
DISCUSSION

4.1. Safety testing

In the clinical study the safety of Latrobe valley potassium humate^{TM} , at a daily dose of 1.8g, has been established, with no adverse events reported or observed. All haematology and biochemical parameters were within normal ranges and no statistically significant differences were detected when the placebo and treated groups were compared, thus leading to the conclusion that the potassium humate has no adverse effects when taken at this dose for 30 days.

4.2. Total symptom score

Although the total symptom score indicated no significant differences when comparing the treated and placebo groups respectively, a trend of decreasing scores was observed for both groups leading to the deduction that even though the trial was purposely run in and out of the pollen season, to take in account fluctuations in pollen count, both groups' symptoms were alleviated. A second trial is planned which will be restricted to the in-season period to eliminate pollen fluctuation.

4.3. Skin prick testing

When the allergen is introduced into the skin on a previously sensitized individual, IgE molecules on the surface of a mast cell are bridged and degranulation of the mast cell occurs. Pre-formed granules containing histamine are released followed by progressive infiltration of the dermis by eosinophils and neutrophils which have been attracted to the site by chemotactic factors. Some agents however may induce mast cell histamine release by non-IgE mediated mechanisms.

With statistical analysis, using an ANOVA and adjusting for baseline, a significant difference was observed with a $p < 0.05$ when comparing the before and after results of the treated versus placebo groups respectively, with 70% of the treated participants

having a decreased wheal at their last visit versus that of the 65% of the placebo group. The reduction in the placebo group could be due to the fact that the trial was run in and out of the pollen season, which was done in order to compensate for the fluctuations in pollen and not wanting this to be a determining factor in the study.

When comparing the flare measurements, which is an inflammatory response to an allergen, it was demonstrated that there is a significant difference between the two groups, and when adjusting for baseline the treated groups flare measurements had significantly decreased. Hereby proving that potassium humate operates as an anti-inflammatory agent.

4.4. Eosinophils

The involvement of eosinophils is a trademark of allergic rhinitis. The accumulations of the cells to local tissue sites include the effects of cytokines and other cellular and humoral mediatiors (Gleich *et al*., 1994). Eosinophils are rarely present within nasal mucosa of individuals who are non-atopic and have no nasal symptoms. Increases of eosinophils are evident both within the lamina propria and epithelium of the nasal mucosa (Breddin *et al*., 1993, 1995, Bentley *et al*., 1992, Jacobcon *et al*., 1999), and their recovery is also increased in nasal luminal samples (Andersson *et al*., 1989, Pipkom *et al*., 1988, Salib *et al*., 2005).

Elevated concentrations of the eosinophil granule components, ECP and eosinophil protein X (EPX), as well as eosinophil peroxidase have been identified in nasal lavage of patients with active seasonal allergic rhinitis in comparison with that of both non-rhinitic subjects and the same seasonal rhinitics out of season (Wilson *et al*., 1998, Ahlstrom-Emanuelsson *et al*., 2004, Klimek *et al*., 1996, Lorenzo *et al*., 1997, Svensson *et al*., 1990, Wilson *et al*., 1998). The ECP levels in nasal lavage have been found to correlate with both the number of eosinophils (Andersson *et al*., 1989, Miller et al., 1982, Wilson *et al*., 1998, Bickmore 1978), consistent with the recruitment and activation of eosinophils in this disease, and symptom score (Ahlstrom-Emanuelsson *et al*., 2004).

After the statistical analysis was done, using an ANOVA and adjusting for baseline, a significant difference was observed with a $p < 0.05$ when comparing the before and after results of the treated versus placebo groups respectively, with 55% of the treated participants having a decreased eosinophil grading with there last visit versus that of the 5% of the placebo group.

The recruitment and activation of eosinophils is considered critical to clinical disease expression, through the ability of these cells, when activated, to release mediators that induce development of inflammation. So in decreasing the recruitment of eosinophils it proves the anti-allergic effect of potassium humate on yet another surrogate marker of allergic rhinitis but due to there being no significance when evaluating the total symptom scores, the actual alleviation of the symptoms is still yet to be proven.

When comparing the smears of the two groups it was found that there is a significant difference between the two groups showing that when adjusting the results for baseline the treated groups' nasal eosinophil recruitment had significantly decreased, which correlates with other therapies for hay fever (Di Lorenzo *et al*., 2004).

4.5. Neutrophils

Neutrophils can contribute to the inflammation by releasing their own chemokines, superoxide anion and granular enzymes. Neutrophil elastase (NE) in fluids in the epithelial lining of the airways has been reported to destroy structures of the extracellular matrix, induce further inflammatory cytokine release, and produce clinical symptoms (Teran *et al*., 1997; Nadel *et al*., 1999; Gadek *et al*., 1979). Neutrophils can also produce a wide range of products including lipids, proteases, microbicidal products, reactive oxygen intermediates (superoxide, H_2O_2, OH^-) and nitric oxide (Sampson, 2000).

These cytokines are likely to add to neutrophil recruitment and hyper-responsiveness. However, because of their potential to release large amounts of histotoxic and other pro-inflammatory agents, this cell also has the capacity to produce significant tissue injury. Hence inappropriate or excessive activation of neutrophils in vivo has been

implicated in the pathogenesis of a wide variety of inflammatory diseases (Brazil *et al*., 2005).

The neutrophil adhesion assay measures the amount of neutrophils that adhered to the BHK 238-1 and 331-7 cell lines respectively. The BHK 331-7 contains ICAM-1 and is therefore more specific for the binding of neutrophils to ICAM-1. Both stimulated and resting cells were measured. Analysing MPO release quantitated adherent neutrophils.

As seen and calculated from the results, it was clearly indicated that there were no significant differences between the stimulated and resting cells as well as no significant difference with relation to neutrophil adhesion with an increasing dosage range of potassium humate treatment. This was observed for both the BHK 238-1 cell line as well as the BHK 331-7 cell line respectively. This hereby indicates that the potassium humate had no influence on the adhesion of neutrophils both stimulated or resting and thus would have no effect on the amount of MPO released and in turn having no effect on the inflammatory response with respect to neutrophil adhesion.

4.6. Basophils

As new tools for basophil purification, detection in blood and monitoring activation by fluorescence-activated cell sorting have been developed and marketed, there is now a new surge of interest in this cell type, documented by the increasing number of publications and laboratories addressing the immunobiology of basophils.

Thus basophils are now much more amenable to research, and as a result the perception of their role in the immune system has shifted from mere effector cells, involved in allergic reactions by virtue of their high-affinity immunoglobulin E (IgE) receptor activation dependent release of leucotrienes and histamine, to a cell which is potentially deeply implicated in modulating the immune responses to parasites and allergens (Irani *et al*., 1989, Walls *et al*., 1990).

When analyzing the data using the difference between the positive and the test and then also the difference between the negative and the test using an ANOVA and

adjusting for baseline no significant differences were observed when comparing the placebo group with that of the treated group with respect to there before and after results. 64.71% of the placebo group experienced a reduction in basophils stimulation compared to 35.71 % of the treated group, this is probably due to uncontrolled circumstances where the whereabouts and means of arrival of the patients to site are unknown and could lead to the patients' basophils being already stimulated. This should be taken into account if this method were to be used in similar future studies.

Thus concluding that potassium humate, at this dose, may not have an effect on basophil stimulation and thus histamine release, and could therefore have a more specific anti-inflammatory effect, but this is not definite due the uncontrolled circumstances as mentioned above.

4.7. Cytokines

Studies report that T helper (Th) cells subdivide into mutually exclusive Th1 (producing mainly IL-2 and IFN-γ) and Th2 (producing mainly IL-4, IL-5, IL-9, IL-10 and IL-13) cells. These two subsets act together with cells that exhibit an unrestricted cytokine profile (namely Th0) (Mosmamm *et al*., 1986, 1989, 1996, Cherwinski *et al*., 1987). Th1 cells are primarily involved in cell-mediated immune responses, whereas Th2 cells fulfil an important role in humoral and allergic immune responses (Mosmamm *et al*., 1996).

A T helper (Th) 1/Th2 cytokine imbalance with a predominance of Th2 cytokines has been suggested of pathogenic importance in AR. Th2 cells fulfil an important role in humoral and allergic immune responses, especially through the production of IL-4, IL-5 and IL-10. Th2 cells can be readily found in the late-phase response in the lung and after intradermal injection of antigens into the skin. Some of the cytokines secreted by these cells have direct pro-inflammatory effects; both IL-4 and IL-5 promote the recruitment and survival of esosinophils and mast cells (Mosmamm *et al*., 1986, 1989, 1996, Cherwinski *et al*., Romagnani 1997).

IL-8 and MPO Levels are higher in individuals suffering from chronic rhinosinusitis. IL-8 recruits and activates neutrophils in sinus tissue. Nasal epithelial cells,

neutrophils, mast cells, fibroblasts, for example, release eotaxin, IL-1β, IL-8 and TNF-α, which can induce influx and activation of eosinophils (Bradding *et al*., 1995, Abdelaziz *et al*., 1998, Terada *et al*., 2000).

Even though when analysing the data for both the Th1 and Th2 cytokines using an ANOVA and adjusting for baseline, no significant difference were observed when comparing the treated vs the placebo group, certain correlations and trends were observed. With respect to IL-4 and IL-5, which cause influx of activated eosinophils and promotes IgE production respectively, the mean of the placebo group increased where as that of the treated group decreased, correlating with the results of nasal eosinophil staining and grading result. Hereby confirming that the decreased amount of nasal eosinophils could be as a result of the decreased expression of the IL-4 and IL-5 cytokines.

With respect to IL-8 and IL-1β, both these cytokines had a decreased secretion in the treated group versus the increased expression of the placebo group. This too could be responsible for the decreased recruitment of eosinophils and a decreased secretion of IL-8 also leads to a decreased recruitment and activation of neutrophils, which also leads to decreased inflammation.

Due to the large variation in some of the cytokine tests, which are attributed to unknown or uncontrolled events, this made it very hard to interpret the results.

5. Conclusion

Although a significant reduction was seen in the number of eosinophils present in the nasal smears as well as a reduction in the size of the wheals that developed after the allergen challenge in the group using the potassium humate, no conclusion could be made on the efficacy of the potassium humate as an antihistaminic agent.

This might be different if the study period is restricted to the months during which the pollen count is at its highest. A higher dose of the potassium humate may also deliver more positive anti-allergic results. Because very limited information and knowledge is available on the properties, mechanism of action, function and uses of potassium humate, a broad spectrum of assays and tests have been and are currently being worked on in order to pinpoint the possible uses and mechanisms of action of potassium humate.

A rat model of contact hypersensitvity has shown great promise in using potassium humate as an antí-inflammatory agent (Van Rensburg *et al*., in press). This study was confirmed by the significant decrease in the skin prick test results indicating that potassium humate possesses anti-inflammatory properties. With regard to it's mechanism of action, preliminary results obtained in this study indicate that potassium humate might decrease the recruitment and activation of eosinophils by depressing the expression of IL-4, IL-5, IL-8 and IL-1β.

In order to confirm these results and have a greater understanding of the mechanism of action of both the anti-inflammatory and anti-allergic properties of potassium humate, more in depth work should be carried out focusing on the cytokines in question.

6. References

Abdelaziz MM, Devalia JL, Khair OA. (1998) Effect of fexofenadine on eosinophil-induced changes in epithelial permeability and cytokine release from nasal epithelial cells of patients with seasonal allergic rhinitis. *J Allergy ClinImmunol* 101: 410-420.

Ahlstrom-Emanuelsson CA, Greiff L, Andersson M, Persson CGA, Erjefalt JS. (2004) Eosinophil degranulation status in allergic rhinitis: observations before and during seasonal allergen exposure. *Eur Respir J*. 24:750–7.

Andersson M, Andersson P, Venge P, Pipkorn U.(1989) Eosinophils and eosinophil cationic protein in nasal lavages in allergen-induced hyperresponsiveness: effects of topical glucorticosteroid treatment. *Allergy* 44:342–8.

Andersson M, Svensson C, Andersson P, Pipkorn U. (1989) Objective monitoring of the allergic inflammatory response of the nasal mucosa in patients with hay fever during natural allergen exposure. *Am Rev Respir Dis* 139:911–4.

Arock M, Merle-Beral H, Dugas B. (1993) IL-4 release by human leukemic and activated normal basophils. *J Immunol* 151: 1441-1447.

Baatz H. (1988) Moortherapie en der Frauenheilkunde., *Moortherapie: Grundlagen und Anwendungen,* Flaig, W., Goecke, C. and Kauffels, W. 161 - 168, Ueberreuter, Wien-Berlin.

Bachert C, Wagenmann M, Hauser U. (1995) Proinflammatory cytokines: measurement in nasal secretion and induction of adhesion receptor expression. *Int Arch Allergy Immunol* 107: 106-108.

Benson M, StrannegaÊrd I-L, Wennergren G. (1997) Cytokines in nasal fluids from school children with seasonal allergic rhinitis. *Ped Allergy Immunol* 8:1 43-149.

Benson M, StrannegaÊrd IL, StrannegaÊrd OÈ. (2000) Topical steroid treatment of allergic rhinitis decreases nasal fluid TH2 cytokines, eosinophils, eosinophil cationic protein, and IgE but has no significant effect on IFN-gamma, IL-1beta, TNFalpha, or neutrophils. *J Allergy Clin Immunol* 106: 307-312.

Bentley AM, Jacobson MR, Cumberworth V *et al*. (1992) Immunohistology of the nasal mucosa in seasonal allergic rhinitis: increases in activated eosinophils and epithelial mast cells. *J Allergy Clin Immunol*. 89:877–83.

Berman D, Toerien A. (1991) Analysis of eosinophils. THE ALLSA HANDBOOK OF PRACTICAL ALLERGY. Appendix xii.

Bernard A, Brottier P, Georget E, Lepage V, Boumsell L. (1984) Joint report of the first international workshop on human leucocyte differentiation antigens by the investigators of the participating laboratories. Leucocyte Typing I, Bernard, A. et al., Springer Verlag, 9-135.

Bickmore JT. (1978) Nasal cytology in allergy and infection. ORL. *Allergy.* 40:39–46.

Borowitz M, Bauer KD, Duque RE, Horton AF, Marti G, Muirhead KA, Peiper S, Rickman W. (1998) Clinical applications of flow cytometry: Quality assurance and immunophenotyping of lymphocytes; approved guideline. *NCCLS*, 21, 18.

Böyum A. (1968) Isolation of leucocytes from human blood. Further observations: Methylcellulose, dextran, and ficoll as erythrocyte aggregating agents. *Scand J Clin Lab Invest.* 21: 31-50.

Bradding P, Feather IH, Wilson S, Bardin P, Holgate ST, Howarth PH. (1993) Immunolocalisation of cytokines in the nasal mucosa of normal and perennial rhinitis subjects: the mast cell as a source of IL-4, IL-5 and IL-6 in human allergic inflammation. *J Immunol.* 151:3853–65.

Bradding P, Feather IH, Wilson S, Holgate ST, Howarth PH. Cytokine immunoreactivity in seasonal rhinitis: regulation by a topical corticosteroid. (1995) *Am J Resp Crit Care Med.* 151: 1900–6.

Bradding P, Mediwake R, Feather IH. (1995) TNF alpha is localized to nasal mucosal mast cells and is released in acute allergic rhinitis. *Clin Exp Allergy* 25:406-415.

Brazil TJ, Dagleish MP, McGorum BC, Dixon PM, *et al*. (2005) Kinetics of pulmonary neutrophil recruitment and clearance in a natural and spontaneously resolving model of airway inflammation. *Clin Exp Allergy.* 35:854–865.

Brunner T, Heusser CH, Dahinden CA. (1993) Human peripheral blood basophils primed by interleukin 3 (IL-3) produce IL-4 in response to immunoglobulin E receptor stimulation. *J Exp Med* 177: 605-611.

Bühring HJ, Simmons PJ, Pudney M, Müller R, Jarrossay D, van Agthoven A, Willheim, M, Brugger W, Valent P, Kanz L. (1999) The monoclonal antibody 97A6 defines a novel surface antigen expressed on human basophils and their multipotent and unipotent progenitors. *Blood* 7: 94, 2343-2356.

Bühring HJ. (2002) E-NPP3 (CD203c). Leucocyte typing VII, Section New CD antigens, MC10, Oxford University Press, 377-378.

Cameron L, Hamid Q, Wright E. (2000) Local synthesis of epsilon germline gene transcripts, IL-4, and IL-13 in allergic nasal mucosa after ex vivo allergen exposure. *J Allergy Clin Immunol* 106: 46-52.

Carter LL, Swain SL. (1997) Single cell analyses of cytokine production. *Curr. Opin. Immunol* 9: 177-182.

Casale TB, Costa JJ, Galli SJ. (1997) TN alpha is important in human lung allergic reactions. *Am J Respir Cell Mol Biol.* 15:35-44.

Cherwinski H, Schunacher JH, Brown KD, Mosmann TR. (1987) Two types of mouse helper T cell clone. *J Exp Med*. 166:1229–44.

Cosmi L, Annunziato F, Iwasaki M, Galli G, Manetti R, Maggi E, Nagata K, Romagnani S. (2000) CRTH2 is the most reliable marker for the detection of circulating human type 2 Th and type 2 T cytotoxic cells in health and disease. *Eur. J. Immunol*. 30: 2972-2979.

Cosmi L, Annunziato F, Maggi E, Romagnani S, Manetti R. (2001) Chemoattractant Receptors expressed on Type 2 T cells and their role in disease. *Int. Arch Allergy Immunol* 125: 273-279.

Cosmi L, Annunziato F, Galli G, Manetti R, Maggi E, Romagnani S. (2001) CRTH2: marker for the detection of human Th2 and Tc2 cells. *Progress in Basic and Clinical Immunology* 2001: 25-29.

Di Lorenzo G, Pacor ML, Pellitteri ME *et al*. (2004) Randomised placebo-controlled trial comparing fluticasone aqueous nasal spray in mono-therapy, fluticasone plus cetirizine, fluticasone plus montelukast and cetirizine plus montelukast for seasonal allergic rhinitis. *Clin Exp Allergy* 34:259–67.

Dressler LG. (1997) Specimen handling, storage, and preparation. Curr. *Protocols Cytometry.* Chapter 5, 5.0.1-5.2.15.

Durham SR, Ying S, Varney VA. (1996) Grass pollen immunotherapy inhibits allergen-induced infiltration of CD41 T lymphocytes and eosinophils in the nasal mucosa and increases the number of cells expressing mRNA for interferon-gamma. *J Allergy Clin Immunol* 97: 1356-1365.

Ferrara G, Loffredo E, Simeone R. Senesi N. (2000) Evaluation of the antimutagenic and desmutagenic effects of humic and fulvic acid on roof tips of Vicia faba. *Environ Toxicol* 15:513-517.

Gadek JE, Kelman JA, Fells G, *et al.* (1979) Collagenase in the lower respiratory tract of patients with idiopathic pulmonary fibrosis. *N. Engl. J. Med.* 301: 737–42.

Galli SJ. (2000) Mast cells and basophils. *Curr. Opin. Hematol* 7: 32-39.

Gibbs BF, Haas H, Falcone FH. (1996) Purified human peripheral blood basophils release interleukin-13 and preformed interleukin-4 following immunological activation. *Eur J Immunol* 26: 2493-2498.

Gibson PG, Manning PJ, O'Byrne PM, *et al*. (1991) Allergy-induced asthmatic response. *Am Rev Pespir Dis*. 143: 331-335

Gleich GJ, Adolphson CR, Leiferman KM. (1993) The biology of eosinophilic leukocyte. *Annu Rev Med 44*: 85-101.

Gleich GJ, Kay AB, *et al*. (1994) Eosinophils in allergy and inflammation. New York: Marcel Dekker. 1-24: 1-496.

Håkansson L, Nielsen LS, Teder P. (1994) Measurement of neutrophil and eosinophil adhesion to E-selectin, VCAM-1, and ICAM-1 by the use of transfected fibroblast cell lines. *J Immun Methods* 176: 53-66.

Haanel BF. (1942) Facts about peat. Mines Branch Publication No. 614

Hannet I, Erkeller-Yuksel F, Lydyard P, Deneys V, DeBruyère M. (1992) Developmental and maturational changes in human blood lymphocyte subpopulations. *Immunol. Today* 6: 13, 215-218.

Hirai H, Tanaka K, Yoshie O, Ogawa K, Kenmotsu K, Takamori Y, Ichimasa M, Sugamura K, Nakamura M, Takàno S, and Nagata K. (2001) Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. *J. Exp. Med*. 2: 193, 255-261.

Hirai H, Tanaka K, Takano S, Ichimasa M, Nakamura M, and Nagata K. (2002) Cutting Edge: Agonistic Effect of Indomethacin on a Prostaglandin D2 Receptor, CRTH2. *J. Immunol*. 168: 981-985.

Holgate ST. (1999) J Allergy *Clin Immunol* 104: 1139-1146.

Holgate ST. (2000) Epithelial damage and response. *Clin Exp Allergy* 30: 37-41

Holgate ST. (2000) The role of mast cells and basophils in inflammation. *Clin. Exp. Allergy* 30: 28-32

Iubitskaia NS. Ivanov EM. (1999) Sodium humate in the treatment of osteoarthrosis patients. *Vopr Kurortol Fizioter Lech Fiz Kult* 5; 22-24.

Jacobson MR, Juliusson S, Lowhagen O, Balder B, Kay AB, Durham SR.(1999) Effect of topical corticosteroids on seasonal increases in epithelial eosinophils and mast cells in allergic rhinitis: a comparison of nasal brush and biopsy methods. *Clin Exp Allergy*. 29:1347–55.

Jansen van Rensburg C, Dekker J, van Rensburg CEJ, van Ryssen JBJ. (2002) The effect of oxihumate on aflatoxicosis in broilers. Joint Congress of the Grassland Society of Southern Africa and the South African Society for Animal Science 13-17 May, Christiana Aventura.

Johnston SL, Holgate ST. (1990) Cellular and chemical mediators their roles in allergic diseases. *Curr. Opin. Immunol* 2: 513-524.

Jooné GK, Dekker J, van Rensburg CEJ. (2001) An *in vitro* investigation of the anti-inflammatory properties of oxihumate.11th International Congress of Immunology. Sockholm, Sweden. 22-27 July.

Jooné GK, Dekker J, van Rensburg CEJ. (2003) Investigation of the immunostimulatory properties of oxyhumate. *Zeitschrift fur Naturforschung Redaktion.* 58c: 263-267.

Kallus J. (1964) Gastritis, das gastroduodenale Ulkus und Neydhartinger Moor. *Bericht über den 8. Internationalen Kongress für universelle Moor- und Torfforschung, Länderverlag* 112-114.

Kelginbaev NS, SorokinaVA, Stefanidu AG, Ismailova VN. (1994) Treatment of long tubular bone fractures with Mumie astil preparations in experiments and clincal conditions. *Exp Surg Anesthes* 18: 31-35.

Kleinschmidt J. (1988) Moortherapie bei rheumatischen Erkrankungen., *Moortherapie: Grundlagen und Anwendungen,* Flaig, W., Goecke, C. and Kauffels, W. 216 - 224, Ueberreuter, Wien-Berlin.

Klimek L, Riechelmann H, Amedie R. (1996) Eosinophil cationic protein in nasal secretions and blood-serum in grass-pollen allergic rhinitis. *Am J Rhinol.* 10:319–22.

Klimek L, Dorman D, Jarman ER. (1999) Short-term preseasonal birch pollen allergoid immunotherapy influences symptoms, specific nasal provocation and cytokine levels in nasal secretions, but not peripheral T-cell responses in patients with allergic rhinitis. *Clin Exp Allergy* 29: 1326-1335.

Kovarik R. (1988) Über die Anwendung von Präparaten aus Torf, bzw. Huminstoffen bei gynäkologischen Erkrankungen., *Moortherapie: Grundlagen und Anwendungen.*

Kroegel C, Warner JC, Virchow JR, Matthys H. (1994) Pulmonary immune cells in health and disease: the eosinophil leukocyte (Part II). *Eur Respir J 7*: 743-760.

Lent W. (1988), Bericht über die Moorforschung und anwendung in der DDR, Polen, Tschechoslowakei und UdSSR., *Moortherapie: Grundlagen und Anwendungen,* Flaig.

Li H, Sim TC, Alam R. (1996) IL-13 released by and localized in human basophils. *J Immunol* 156: 4833-4838.

Linden M, Greiff L, Andersson M. (1995) Nasal cytokines in common cold and allergic rhinitis. *Clin Exp Allergy* 25: 166-172.

Lorenzo G, Mansueto P, Melluso M *et al*. (1997) Allergic rhinitis to grass pollen: measurement of inflammatory mediators of mast cell and eosinophils in native nasal fluid lavage and in serum out of and during pollen season. *J Allergy Clin Immunol.* 100:832–7.

Martin RJ, Cicutto LC, Smith HR, Ballard RD, Szefler SJ. (1991) Airways infammation in nocturnal asthma. *Am Rev Respir Dis.* 143:351-7.

Marx G, Heumann KG. (1999) Mass-spectrometric investigation of the kinetic stability of chromium and copper complexes with humic substances by isotope-labelling experiments. *Fresenius' J Anal Chem* 364; 489-494.

Miller RE, Paradise JL, Friday GA, Fireman P, Voitin D. (1982) The nasal smear for eosinophils: its value in children with seasonal allergic rhinitis. *Am J Dis Child.* 136:1009–11.

Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. (1986) Two types of murine helper T cell clone. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol.* 136:2348–57.

Mosmann TR, Coffman RL. (1989) Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol*. 7:145–73.

Mosmann TR, Sad S. (1996) The expanding universe of T cell subsets. Th1, Th2 and more. *Immunol Today*. 17:138–46.

Nadel JA, Takeyama K, Augusti C. (1999) Role of neutrophil elastase in hypersecretion in asthma. *Eur. Respir. J.* 13: 190–6.

Nagata K, Tanaka K, Ogawa K, Kemmotsu K, Imai T, Yoshie O, Abe H, Tada K, Nakamura M, Sugamura K, Takano S. (1999) Selective expression of a novel surface molecule by human Th2 cells in vivo. *J. Immunol*. 162: 1278-1286.

Nagata K, Hirai H, Tanaka K, Ogawa K, Aso T, Sugamura K, Nakamura M, Takano S. (1999) CRTH2, an orphan receptor of T-helper-2-cells, is expressed on basophils and eosinophils and responds to mast cell-derived factor(s). *FEBS Letters* 459: 195-199.

Nielsen T, Helweg C, Siigur K, Kirso U. (1997) Application of HPLC capacity coefficients to characterize the sorbtion of polycyclic aromatic compounds to humic acid *Talanta* 44;1873-1881.

Nifant'eva TI, Shkinev VM, Spivakov BY, Burba P. (1999) Membrane filtration studies of aquatic humic substances and their metal species: a concise overview. Part 2. Evaluation of conditional stability constants by using ultrafiltration. *Talanta* 48; 257-267.

Ochensberger B, Daepp GC, Rihs S, Dahinden CA. (1996) Human blood basophils produce interleukin-13 in response to IgE-receptor-dependent and -independent activation. *Blood* 88: 3028-3037.

Pawankar RU, Okuda M, Suzuki K. (1996) Phenotypic and molecular characteristics of nasal mucosal gd T cells in allergic and infectious rhinitis. *Am J Respir Crit Care Med* 153: 1655-1665.

Pipkorn U, Karlsson G, Enerback L. (1988) The cellular response of the human allergic mucosa to natural allergen exposure. *J Allergy Clin Immuno.l* 82:1046–54.

Platz IJ, Binder M, Marxer A, Lischka G, Valent P, Buhring HJ. (2002) Hymenoptera-venom-induced upregulation of the basophil activation marker ecto-nucleotide pyrophosphatase/phosphodiesterase 3 in sensitized individuals. *Int Arch Allergy Immunol* 126: 4, 335-42.

Prosen H, Zupancic-Krajl L. (2000) The interaction of triazine herbicides with humic acids. *Chromatographia* 51 (suppl);S155-S164.

Romagnani S. (1995) Biology of human TH1 and TH2 cells. *J. Clin. Immunol* 3, 15: 121-129.

Romagnani S.(1997) The Th1/Th2 paradigm. *Immunol Today.* 8:263–6.

Romagnani S. (2000) The role of lymphocytes in allergic disease. *Allergy Clin Immunol* 105: 399-408.

Sala A, *et al*. (1997) The polymorphonuclear leukocyte: a cell tuned for transcellular biosynthesis of cys-leukotrienes. *J Physiol Pharmacol.* 48:665-73.

Salib R, Lau L, Howarth PH. (2005) Nasal lavage fluid concentrations of eotaxin-1 in naturally occurring allergic rhinitis: relationship to disease activity, nasal luminal eosinophil influx, and plasma protein exudation. *Clin Exp Allergy.* In press.

Salvi S, Holgate ST. (1999) Could the airway epithelium play an important role in mucosal immunoglobulin A production? Clin Exp Allergy 29: 1597-1605.

Sampson AP. (2000) The role of eosinophils and neutrophils in inflammation. *J. Clin and Exp. Allergy.* 30: 22-27.

Sander B, Anderson J, Anderson U. (1991) Assessment of cytokines by immunofluorescence and the paraformaldehyde-saponin procedure. *Immunol. Rev* 119: 65-93.

Sauvant MP, Pepin, D and Guillot J. (1999) Effects of humic substances and phenolic compounds on the in vitro toxicity of aluminium. *Ecotoxicology and Evironmental Safety* 44: 47-55.

Shanmukhappa H. and Neelakantan K. (1990) Influence of humic acid on the toxicity of copper, cadmium and lead to the unicellular alga, *Synechosystis Aquatills*. *Bull Environment Contam Toxicol* 44: 840-843.

Shepetkin I, Khlebnikov A., Kwon BS. (2002) Medical drugs from humus matter: Focus on mumie. *Drug Development Research* 57: 150-159.

Shores EW, Love PE. (1997) TCR ζ-chain in T cell development and selection. *Cur. Opin. Immunol 9*: 380-389.

Sim TC, Grant JA, Hilsmeier KA. (1994) Proinflammatory cytokines in nasal secretions of allergic subjects after antigen challenge. *Am J Respir Crit Care Med* 149:339-344.

Smith HR, Larsen GL, Cherniack RM, *et al*. (1992) Infammatory cells and eicosanoid mediators in subjects with late asthmatic responses and increases in airway responsiveness. *J Allergy Clin Immunol.* 89:1076-84.

Soliev TS. (1983) The treatment of deforming osteoarthrosis by non-specific bio-stimulator Mumie. *Med J Uzcek* 8: 19-21.

Stackhouse RH. and Benson WH. (1989) The effect of humic acid on the toxicity and bioavailability of trivalent chromium. *Ecotoxicology and Evironmental Safety* 17: 105-111.

Stankova J, Rola-Pleszczynski M. (1992) Leukotriene B4 stimulates c-fos and c-jun gene transcription and AP-1 binding activity in human monocytes. *Biochem J* 282:625-9.

Stelzer GT, Marti G, Hurley A, McCoy P Jr, Lovett EJ, Schwartz A. (1997) U.S. Canadian consensus recommendations on the immunophenotypic analysis of hematologic neoplasia by flow cytometry: Standardization and validation of laboratory procedures. *Cytometry*. 30: 214-230.

Suleimanov I. (1972) Effects of Mumie on bone regeneration in paatients subjected to surgery for osteoarticular tuberculosis. *Ortop Travm Protez* 33: 64-66.

Svensson C, Andersson M, Persson CG, Venge P, Alkner U, Pipkorn U. (1990) Albumin, bradykinin and eosinophil cationic protein on the nasal mucosal surface in patients with hay fever during natural allergen exposure. *J Allergy Clin Immunol.* 85: 828–33.

Terada N, Hamano N, Nomura T. (2000) Interleukin-13 and tumour necrosis factor-alpha synergistically induce eotaxin production in human nasal fibroblasts. *Clin Exp Allergy* 30: 348-355.

Teran LM, Carroll M, Frew AJ, *et al*. (1995) Neutrophil infux and interleukin-8 release after segmental allergen or saline challenge in asthmatics. *Int Arch Allergy Immuno.l* 107:374-5.

Teran LM, Johnston SL, Schroder J-M, Church MK, Holgate ST. (1997) Role of nasal interleukin-8 in neutrophil recruitment and activation in children with virus-induced asthma. *Am. J. Respir. Crit. Care Med.* 155: 1362–6.

Thibault G, Bardos P. (1995) Compared TCR and CD3ε expression on αβ and γδ cells. Evidence for the association of two TCR heterodimers with three CD3ε chains in the TCR/CD3 complex. *J. Immunol* 154: 3814-3820.

Tomee JF, van Weissenbruch R, de Monchy JG. (1998) Interactions between inhalant allergen extracts and airway epithelial cells: effect on cytokine production and cell detachment. *J Allergy Clin Immunol* 102: 75-85.

Tunnacliffe A, Olsson C, Traunecker A, Krissansen GW, Karjalainen K, De la Hera A. (1989) The majority of CD3 epitopes are conferred by the ε chain. Leucocyte Typing IV, White Cell Differentiation Antigens. W. Knapp, et al., Eds., Oxford University Press, 295-296.

Uguccioni M, Mackay CR, Ochensberger B. (1997) High expression of the chemokine receptor CCR3 in human blood basophils. Role in activation by eotaxin, MCP-4, and other chemokines. *J Clin Invest* 100: 1137-1143.

Van Agthoven A, Terhorst C, Reinherz EL, Schlossman SF. (1981) Characterization of T cell surface glycoproteins T1 and T3 present on all human peripheral T lymphocytes and functional mature T lymphocytes. *Eur. J. Immunol* 11: 18-21.

Van Beneden G. (1971) Les matières organiques dans les eaux et les agents de alnéothérapie *Presse Therm Clim* 108: 195-204.

Van Rensburg CEJ, Dekker J, Van Rensburg EJ, Weiss R, Schneider J. (2001) An *in vitro* investigation of the anti-HIV properties of oxihumate. International Immunopharmacology Congress. Sun City, South Africa. 26-20 September.

Van Rensburg CEJ, Dekker J, Weiss R, Smith TL, Janse van Rensburg E., Schneider, J. (2002) Investigation of the anti-HIV properties of oxihumate. *Chemotherapy* 48: 138-143.

Van Rensburg CEJ, Joone G, Dekker J. (1999) Evaluation of the immuno-stimulatory properties of oxyhumic acid. *Fatigue 2000: International Conference.* 23-24 April, London.

Varney VA, Jacobson MR, Sudderick RM. (1992) Immunohistology of the nasal mucosa following allergen-induced rhinitis. Identification of activated T lymphocytes, eosinophils, and neutrophils. *Am Rev Respir Dis* 146:170-176.

Wardlaw AJ, Moqbel R, Kay B. (1995). Eosinophil: biology and role in disease, p. 151-266. In *Advances in Immunology* Academic Press. Inc., London.
Weller PF. (1994) Eosinophils: structure and functions. *Curr Opinion Immunol* 6: 85-90.

Weido AJ, Reece LM, Alam R.(1996) Intranasal fluticasone propionate inhibits recovery of chemokines and other cytokinesin nasal secretions in allergen-induced rhinitis. *Ann Allergy Asthma Immunol* 77: 407-415.

Wilson SJ, Lau L, Howarth PH. (1998) Inflammatory mediators in naturally occurring rhinitis. *Clin Exp Allergy*. 28:220–7.

Wilson SJ, Shute JK, Holgate ST. (2000) Localization of interleukin (IL)-4 but not IL-5 to human mast cell secretory granules by immunoelectron microscopy. *Clin Exp Allergy* 30: 493-500.

Yamaoka KA, Kolb JP. (1993) Leukotriene B4 induces interleukin 5 generation from human T lymphocytes. *Eur J Immunol*. 23:2392-8.

Yamaoka KA, Dugas B, Paul-Eugene N, Mencia-Huerta JM, Braquet P, Kolb JP. (1994) Leukotriene B4 enhances IL-4-induced IgE production from normal human lymphocytes. *Cell Immunol.* 156:124-34.

Yanagihara Y, Kajiwara K, Basaki Y. (1998) Cultured basophils but not cultured mast cells induce human IgE synthesis in B cells after immunologic stimulation. *Clin Exp Immunol* 111: 136-143.

Ying S, Durham SR, Jacobson MR. (1994) T lymphocytes and mast cells express mRNA for IL-4 in the nasal mucosa in allergen-induced rhinitis. *Immunol* 82: 200-2006.