Investigating the Effect of the Homeopathic Immunomodulator, MODUL8® on Blood Count, Bronchial Lavage and Fibrin Ultrastructure on Experimental Asthmatic BALB/c Mice

Investigación del Efecto del Inmunomodulador Homeopatico Modul8® sobre Sangre, Lavado Bronquial y Ultraestructura de la Fibrina en Ratones Experimentales Asmáticos BALB/c

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SUMMARY: Modul8® is a composite mixture of natural products that are known to be an immunomodulator. In the current study the effect of this immunomodulator is tested on an experimental asthmatic BALB/c mouse model to investigate its properties on the white blood cell count in the blood and bronchial lavage of the animals since white blood cells play a fundamental role in the inflammatory process involved in asthma. As it is known that platelets also play an important role in the immune system, the ultrastructure of platelets and fibrin networks were also investigated by scanning electron microscopy. The animals were sensitised, nebulized and treated over a period of 43 days until termination. Results from the blood smears as well as the bronchial lavage smears revealed significantly higher eosinophil counts in the asthmatic group compared to the control and treated groups. Changes in the ultrastructure of the platelets and fibrin networks could also be observed, with the Modul8® -treated group appearing similar to that of the control group where thick major and thin minor fibres could clearly be distinguished and a tight mass of platelet aggregate could be observed. Whereas the fibrin networks from the asthmatic animals appeared flimsy with a tight mass of thin fibres covering the thick major fibres. The asthmatic platelet aggregates appeared granular without the tight round appearance of the control platelet aggregates. It is therefore concluded that Modul8® positively influences the white blood cell counts by altering the asthmatic profile to look similar to that of the control. Also, it seems as if Modul8® has a stabilizing effect on the platelets and fibrin networks. From these results it can be suggested that Modul8® might successfully be used in the treatment of inflammatory conditions such as asthma.

KEY WORDS: Modul8®; Asthma; BALB/c mouse model; White blood cells; Fibrin and platelet ultrastructure.

INTRODUCTION

Modul8® is an immunomodulator of natural origin that has been developed in a Modul8® laboratory in Argentina. Modul8® is an aqueous, odorless and colorless solution that contains *Aconitum napellus, Arsenicum album, Asafoetida, Calcarea carbonica, Conium maculatum, Rhus toxicodendron, Thuja occidentalis, Ipecacuanha, Sulfur, Silicea,* and *Phosphorus.*

Because it is suggested that the product might have a modulation effect on the immune system, and it has been suggested that it might be useful in the treatment of asthma, the question arose whether it has the ability to reduce white blood cells in bronchial lavage and blood counts; and also if it might have a stabilizing effect on blood platelet and fibrin morphology. It has been reported previously that asthma cause a changed blood platelet and fibrin network morphology and that herbal anti-inflammatory products typically used in asthma, might stabilise the platelets and fibrin networks (Pretorius *et al.*, 2007).

Animal models have been used over the years to study the pathophysiology of human diseases, including asthma, and provided important information on that. The results obtained from experimental animal models have also been

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used as the basis for many clinical trials (O'Neil *et al.*, 1999). Although there is no animal model that reproduces the exact pathology of human asthma, they play an important role in the development of novel therapies and the understanding of the pathogenesis of the disease (Isenberg-Feig *et al*., 2003). According to Epstein in 2006, antigen-induced mouse allergic asthma is a useful model for testing novel therapeutics and that this model is a reliable, clinically relevant replica of human disease (Epstein, 2006). Because mice allow for a variety of in vivo immunological applications, the murine model in particular can be successfully used to study asthma (Bice *et al.,* 2000).

White blood cells, especially eosinophils have a major contribution to the airway remodelling associated with chronic asthma. Airway hyperresponsiveness, inflammatory infiltrates and structural changes in the airways are all features of asthma (Humbles *et al.,* 2004). Platelets and fibrin also play an important physiological role in allergic processes and immunological mechanisms including those associated with asthma where platelets participate by acting as inflammatory cells as they are releasing mediators, spasmogens and interacting with other inflammatory cell types. Platelets are activated by a number of stimuli and this activation may be due to, amongst others, inflammatory processes (Butenas & Mann, 2002; Camera *et al*., 1999; Lazarus *et al*., 2003).

In the current study the effect of the immunomodulator Modul8®, on white blood counts and white blood cells present in bronchial lavage as well as platelet and fibrin ultrastructure will be investigated by using the murine asthmatic BALB/c mouse model.

MATERIAL AND METHOD

Implementing the BALB/c asthmatic model. Six-week-old (female) BALB/c mice (each of average weight 20g), maintained in the University of Pretoria Biomedical Research Centre and provided OVA-free food (Balanced EpolT mice cubes and pellets, obtained from EPOL- a division of Rainbow Farms PTY LTD, SA) and water ad libitum, were used. Polycarbonate type III cages were obtained from Techniplast. The animals were kept in a barrier unit with a temperature range of 20-24°C and a relative humidity of 40-60% with a twelve hour day light and twelve hour night time. Six mice were housed per cage and autoclaved pinewood shavings were used as embedding material. White facial tissue paper was also added for enrichment. All experimental protocols complied with the

requirements of the University of Pretoria's Animal Use and Care Committee.

Mice were divided into the following groups: Six control mice, six asthmatic mice, six mice exposed to physiologically comparable levels of Modul8® (10%) and six mice exposed to hydrocortisone at a concentration of 100mg/kg.

Sensitization of mice (on day 0 and 5) involved the intraperitoneal injection of 25mg ovalbumin (OVA) (Grade V; Sigma Aldrich) and 2mg Al(OH)3 dissolved in 0.5ml of 0.9% saline solution. All the mice except the control mice were sensitized.

The mice were nebulized twice daily for one hour on days 13 to 15 and again on days 30 to 32 with 1% OVA dissolved in PBS. An inhalation exposure system (IES) (Manufactured by Glas-Col®) was used for the nebulization. Nebulization involved placing the mice inside a stainless steel sire mesh basket, which is divided into five equal compartments. Each compartment held the six animals from the same experimental group. One complete cycle in the IES included a preheat cycle of 15 minutes followed by the nebulization with the OVA for 60 minutes, a cloud decay cycle for 15 minutes and a decontamination cycle also 15 minutes.

Treatment involved the administration of Modul8® and hydrocortisone (as positive control). Hydrocortisone was injected intra-peritoneally, while Modul8® was administered orally. For humans it is generally advised to take 10 drops of Modul8® 3 times a day. And since the average of 10 drops equals approximately 600ml, it can be said that the human takes in 600 ml three times per day. This will equal an amount of 1800 ml per day (1.8ml / day). When the average weight of a human adult is also considered (between 60-70kg: average 65kg): It can therefore be assumed that a normal person that weighs 65kg will take in 1800ul = 1800ml / 65kg. This will imply that the intake is: 28ml/kg. The average weight of a mouse is around 20g (0.02kg). And since it was established that the intake per day is 28ml/kg, it can be calculated that each mouse should receive 0.56ml of the Modul8® per day in order to keep the same values as the human. The first treatment procedures took place on days 15 to 18, 22 to 25 and the last set of treatments were on days 36 to 39.

Blood smears techniques. Blood samples of mice from each group were collected via orbital puncture on the day of termination. Histological blood smears were prepared and stained with Rapid Heamatological stain. A total of hundred leucocytes were counted in each blood smear.

Bronchial lavage techniques. After termination, a small skin incision was made in the skin of each mouse in the ventral of the trachea. The trachea was exposed by blunt dissection and a small transversal incision was made below the larynx. Through the sheath of a 21G venous catheter 0.3 ml of saline was injected into the trachea and aspirated with a syringe. The bronchial lavage fluids collected for the individual groups were pooled, centrifuged for 2 minutes at 1000rpm and smears were made. The smears were stained with Rapid Heamatological Stain. White blood cells were counted under a 100x magnification and up to a hundred cells were counted per slide.

Preparation of fibrin clots. Blood was collected on the day of termination via orbital puncture. 11 ml citrate was added for every 100 ml of blood drawn. Blood was then centrifuged at 1250 rpm for 2 minutes to obtain platelet rich plasma (PRP).

Human thrombin (provided by The South African National Blood Services) was used to prepare the fibrin clots. The thrombin is 20 U/ml and is made up in biological buffer containing 0.2% human serum albumin. When thrombin is added to PRP, fibrinogen is converted to fibrin and intracellular platelet components e.g. transforming growth factor, platelet derived growth factor and fibroblastic growth factor are released into the coagulum.

10 ml of mouse PRP was mixed with 10 ml of human thrombin. The PRP and thrombin mix was immediately transferred with a pipette tip to a 0.2 μm millipore membrane to form the coagulum (fibrin clot) on the membrane. This millipore membrane was placed in a Petri dish on filter paper dampened with PBS to create a humid environment and placed at 37ºC for 10 minutes. A washing process followed where the millipore membranes with the coagula were placed in PBS and magnetically stirred for 60 minutes. This was done to remove any blood proteins trapped within the fibrin network.

Preparation of washed fibrin clot for SEM. Washed fibrin clots were fixed in 2.5% glutaraldehyde in Dulbecco's Phosphate buffered saline (DPBS) buffer with a pH of 7.4 for 1 hour. Each fibrin clot was rinsed three times in phosphate buffer for 5 minutes before being fixed for 1 hour with 1% Osmium tetraoxide (OsO4.) The samples were rinsed three times with DPBS for 5 minutes and dehydrated serially in 30%, 50%, 70%, and 90% and three times with 100% ethanol. The SEM procedures were completed by critical point drying of the material, mounting, coating with ruthenium and examination of the tissue with a JEOL 6000F FEGSEM.

RESULTS

Bronchial lavage. Three bronchial lavage smears were chosen at random, from each of the four experimental groups, for microscopic evaluation. The numbers of each white blood cell type – monocyte, lymphocyte, eosinophil, basophil and neutrophil – were quantified. Statistical analysis of the recorded counts was performed with the aid of NCSS in order to determine if any difference amongst the group's specific white blood cell counts existed. Each white blood cell species was therefore considered separately via a one-way ANOVA or Kruskal-Wallis one-way ANOVA, depending upon whether the necessary assumptions for the parametric test were met or not.

One-way ANOVA's were utilized for the between group comparisons for each type of white blood cell. This was facilitated due to the observations meeting all the necessary assumptions for this parametric tests utilization - the use of equal sample sizes allowed one to ignore the assumption of equal variance. Table I expresses the statistical tests run for each white blood cell species and the outcomes thereof.

Blood counts. Six blood smears were chosen at random, from each of the five experimental groups, for microscopic evaluation. With the use of a light microscope the numbers of each white blood cell species – monocyte, lymphocyte, eosinophil, basophil and neutrophil – were quantified. Statistical analysis of the recorded counts was preformed with the aid of NCSS in order to determine if any difference amongst the group's specific white blood cell counts existed. Each white blood cell species was therefore considered separately via a one-way ANOVA or Kruskal-Wallis one-way ANOVA, depending upon whether the necessary assumptions for the parametric test were met or not.

One-way ANOVA's were utilized for the between group comparisons for the assessment of monocytes, lymphocytes, neutrophils and basophils. This was facilitated due to the observations meeting all the necessary assumptions for this parametric tests utilization - the use of equal sample sizes allowed one to ignore the assumption of equal variance. The eosinophil comparison was conducted with the aid of the non-parametric Kruskal-Wallis one-way ANOVA, as the data did not assume a normal distribution. Table II expresses the statistical tests run for each white blood cell species and the outcomes thereof.

SEM analysis. Figure 1 shows the differences in the ultrastructure of platelets and fibrin networks in the different experimental groups. Figure 1a and b shows control fibrin

networks and a platelet aggregate. Thick major fibers are indicated with Label A and Label B show fine, minor fibers (Fig. 1a). Figure 1b shows a round compact platelet aggregate with pseudopodia (white arrows). Figure 1c shows fibrin networks of asthmatic animals where the minor fibers (Label C) form a thick, matted layer over the major fibers (Label D). Figure 1d shows a platelet aggregate of asthmatic untreated animals, where the aggregates are loosely arranged. Figure 1e shows fibrin networks and platelet aggregates of Modul8® -treated animals. It was seen that Modul8® stabilized both the fibrin networks and platelet aggregates and SEM analysis revealed the morphology to be the same as that of the controls. Platelets and fibrin networks play an important role in asthma and therefore it is an important observation that Modul8® positively influenced the ultrastructure of these structures.
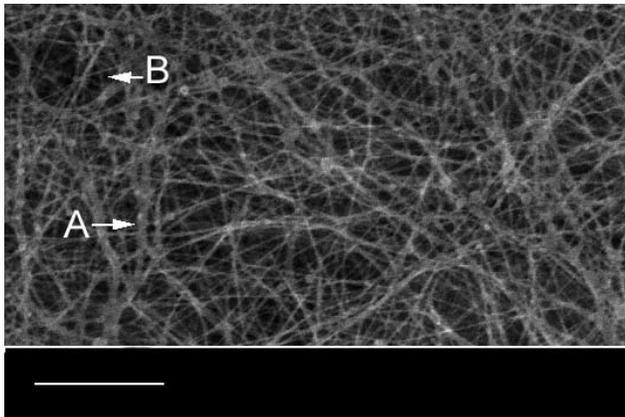

Fig. 1a. Control fibrin network of BALB/c mice with thick, major fibers as well as thin, minor fibers. Label A = thick, major fibers; Label B = thin, minor fibers. Scale bar = 200nm.
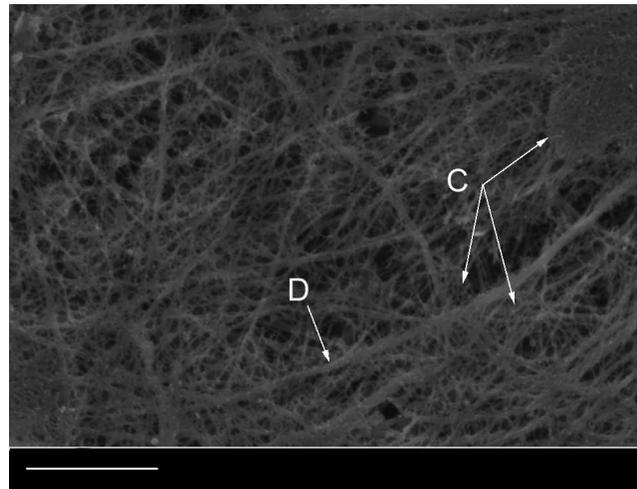

Fig. 1c. Fibrin network of asthmatic BALB/c mice, forming flimsy fibrin network. Label D = matted, thin minor fibers; Label E =. thick, major fibers. Scale bar = 200nm.
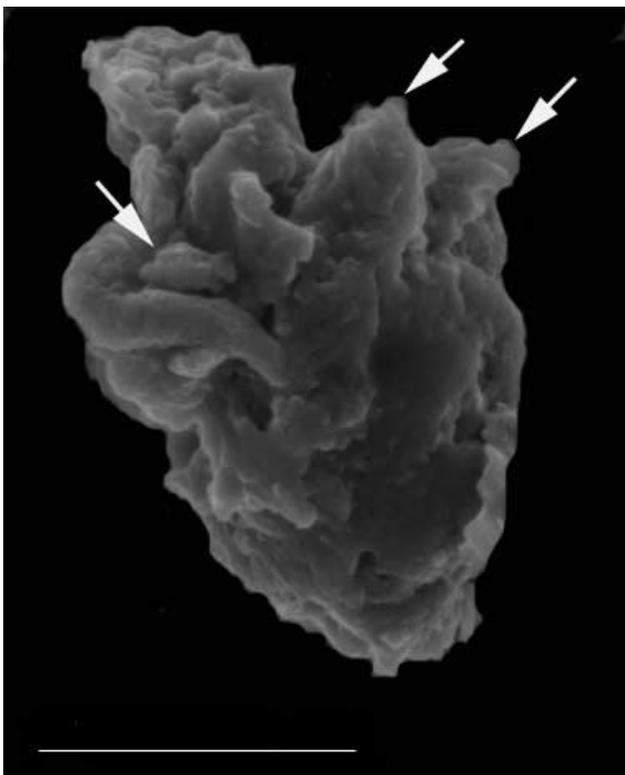

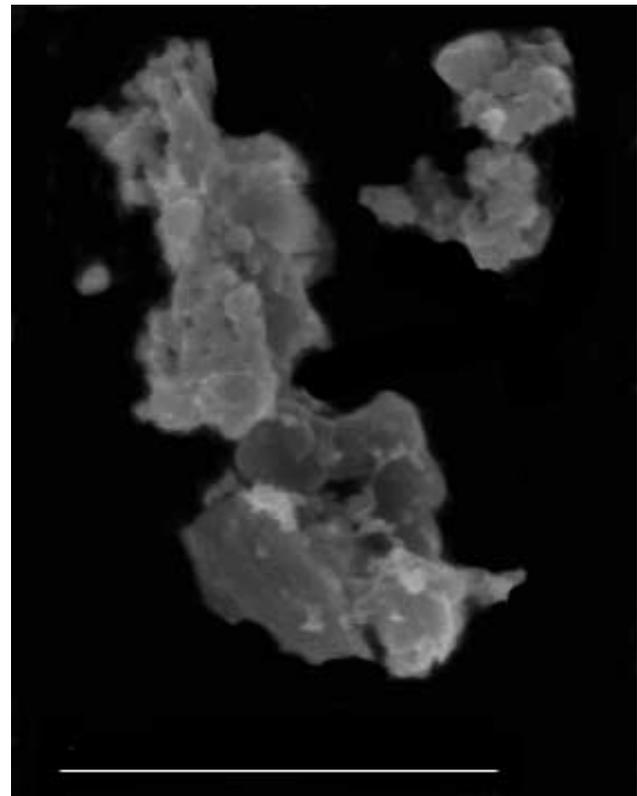Fig. 1b. Control platelet aggregate of BALB/c mice; white arrows – pseudopodia. Scale bar = 1mm.

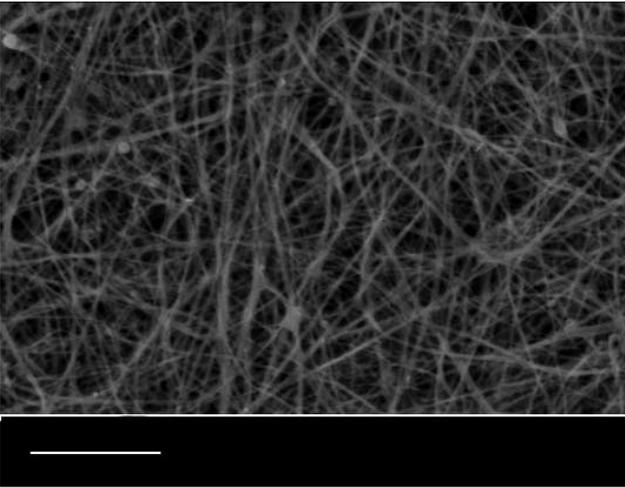Fig. 1d. platelet aggregate of BALB/c mice. Scale bar = 1mm.

Fig. 1e. Fibrin network from asthmatic BALB/c mice treated with Modul8®. Scale bar = 200nm.
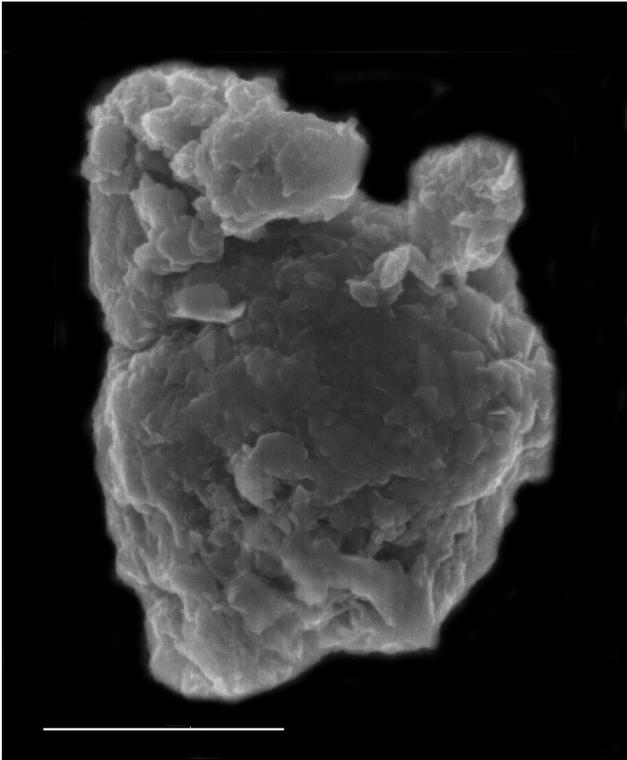

Fig. 1f. Platelet aggregate from asthmatic BALB/c mice treated with Modul8®. Scale bar = 1mm.
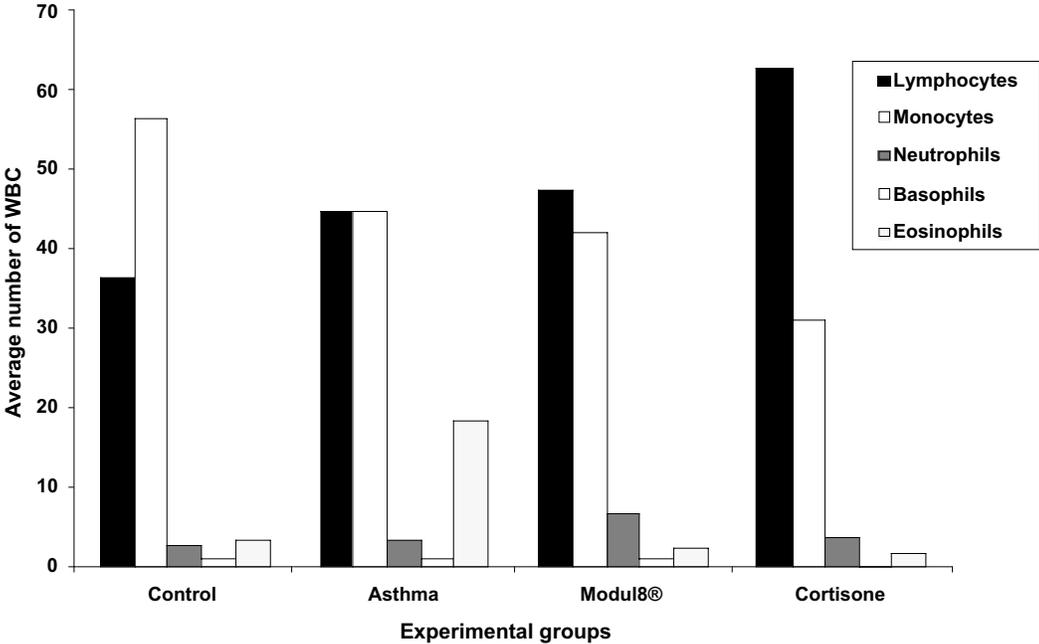

Fig. 2. Average number of the white blood cells counted in the bronchial lavage.
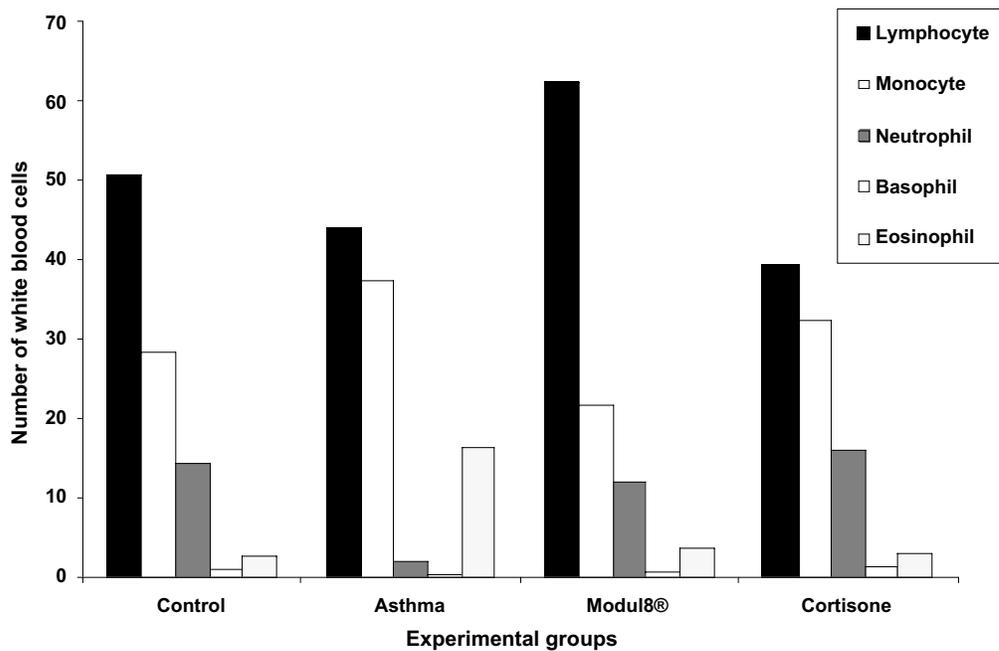
Fig. 3. Average number of white blood cells counted in the blood smears.

Table I. The statistical comparisons preformed upon the various white blood cell species derived from the bronchial lavage of the assessed BALB/c mice

White blood cell species	Test utilized	P value*	Difference between compared groups determined, if applicable, with the aid of Tukey-Kramer Multiple-Comparison Test's
Monocytes	One-way ANOVA	0.0389	The control group possessed a significantly greater monocyte count in comparison to the hydrocortisone group
Lymphocyte	One-way ANOVA	0.244	No significant difference existed between any groups lymphocyte counts
Eosinophil	One-way ANOVA	0.00132	The asthmatic group possessed a significantly greater eosinophil count in comparison to all other assessed groups
Neutrophil	One-way ANOVA	0.551	No significant difference existed between any groups neutrophil counts
Basophil	One-way ANOVA	0.751	No significant difference existed between any groups basophil counts

* Significance was set at a level of 0.05

DISCUSSION

Asthma is a chronic condition characterized by airflow obstruction, airway hyper-responsiveness (AHR) and chronic airway inflammation that usually has an eosinophilic component. Other characteristics associated with this disease include accumulation of eosinophils and CD4 lymphocytes, activation of the epithelium and smooth muscle, mucus hyper-secretion, thickening of the sub-epithelial collagen layer, mast cell degranulation, and smooth muscle hypertrophy and hyperplasia (Kay, 1996). One of the characteristics of asthma in humans or animals is the pulmonary eosinophilia and the progressive tissue damage that is caused by the eosinophilic inflammation (Hogan *et*

Table II. The statistical comparisons preformed upon the various white blood cell species derived from the blood of the assessed BALB/c mice.

White blood cell species	Test utilized	P value*	Difference between compared groups determined, if applicable, with the aid of Tukey-Kramer Multiple-Comparison Test's
Monocytes	One-way ANOVA	0.062	No significant difference existed between any groups monocyte counts
Lymphocyte	One-way ANOVA	0.111	No significant difference existed between any groups lymphocyte counts
Eosinophil	Kruskal-Wallis one-way ANOVA	0.00100	The asthmatic group possessed a significantly greater eosinophil count in comparison to all other assessed groups
Neutrophil	One-way ANOVA	0.00302	The asthmatic group possessed a significantly lower neutrophil count in comparison to all other assessed groups
Basophil	One-way ANOVA	0.217	No significant difference existed between any groups basophil counts

* Significance was set at a level of 0.05

al., 2003). Other characteristics include airway hyper-responsiveness, mucous secretion, and the production of IgE, infiltration of inflammatory cells as well as the release of a number of different mediators (Boulet *et al.,* 1998). Mast cells, eosinophils, T lymphocytes, dendritic cells, macrophages and neutrophils are some of the cells present in the blood smears and bronchial lavage of asthmatic mice. In 2004, Kay *et al.* revealed that eosinophil numbers are always increased in the airways and that these cells are responsible for the release of proteins and growth factors that cause damage to the airway epithelial cells and thereby cause airway remodelling (Kay *et al.,* 2004).

Increased eosinophil and T lymphocyte numbers in the broncho-alveolar lavage fluid are also characteristic of the inflammatory response in patients with asthma (Caramori *et al.*, 2005; Tillie-Leblond *et al.*, 2005; Walker *et al.*, 1991). Up to 90% of the cells in the bronchial lavage of control mice are macrophages. In 2007, Larsen and co-workers also used the BALB/c mouse model and reported that with inflammation the total number of white blood cells increased, but more specifically, the distribution of cells is changed. An increase in eosinophils, neutrophils and sometimes lymphocytes could be observed (Larsen *et al.*, 2007).

Results from the bronchial lavage of the current study revealed a significantly higher monocyte count in the control group compared to the other groups whereas the asthmatic group revealed a significantly higher eosinophil count compared to the other experimental groups. No significant difference could be observed when comparing the average number of lymphocyte, neutrophil and basophil

counts of the different groups (Table I and Fig. 2). Results from the blood smears revealed more or less the same pattern with a significantly higher eosinophil count in the asthmatic group as well as a significantly lower neutrophil count when compared to the other assessed groups. No significant differences could be observed when comparing the lymphocyte, monocyte and basophil counts (Table II and Fig. 3).

Platelet distribution changed significantly in the asthmatic group when compared to the treatment groups, as numerous platelet clumps were found distributed in the bronchial lavage of the asthmatic animals and the absence, or less prominence thereof in the treatment groups could be observed. This observation was noted while doing the blood cell counts during blood smear and bronchial lavage analyses.

Figure 1 shows scanning electron micrographs of the ultrastructure of platelets and fibrin networks in the different experimental groups. Previously it was reported that asthma has an effect on the ultrastructure of platelets and fibrin networks. Pretorius *et al.* in 2007 reported on the ultrastrucuture of platelets and fibrin networks of asthmatic mice compared to that of control mice, hydrocortisone-treated mice and asthmatic mice treated with the plant *Euphorbia hirta.* The results indicated that control mice possess major, thick fibers and minor thin fibers and tight round platelet aggregates could be observed showing typical pseudopodia formation. The minor fibers from the asthmatic animals formed a net covering the major fibers and the platelets appeared as loosely arranged granular aggregates compared to the tight round appearance of the control mice.

Hydrocortisone seemed to influence the fibrin networks by making it more fragile and platelet morphology changed form a tight platelet aggregate to a more granular aggregate, almost similar to that of the asthmatic animals (Pretorius *et al.*). The current research showed that Modul8® stabilized fibrin networks and platelet aggregates. Fibrin networks of Modul8®-treated animals (Figure 1e) appeared similar to that of the controls (Figure 1a) while the fibrin networks of the untreated asthmatic animals (Figure1c) appeared fragile and the thick fibers had a matted covering formed by the thin, minor fibers. Modul8® seem to also stabilize platelet aggregates (Figure 1f) and these aggregates looked the same as the controls (Figure 1b). Asthmatic platelet aggregates (Figure 1d) appeared granular without the tight round appearance of the control platelet aggregates.

The ultrastructure of the platelets and fibrin networks of the Modul8® treated group are similar to that of the control group where thick major and thin minor fibres can clearly be distinguished and a tight mass of platelet aggregate could be observed.

CONCLUSION

During asthma white blood cell counts in bronchial lavage and blood smears are significantly altered. Also, platelet aggregate and fibrin network morphology are changed. It is well-known that platelets and fibrin play and fundamental role in asthma. Also, eosinophils in particular are elevated during the inflammatory response in asthma. In the current research it is seen that Modul8 alters the asthmatic profile of the white blood cell counts and these cell numbers appear similar to that of the controls. Also platelet and fibrin morphology are stabilized and appear similar to that of controls. It is therefore concluded that Modul8® may be used successfully in the treatment of the inflammatory asthmatic condition.

OBERHOLZER, H. M.; VIEIRA, W. A. & PRETORIUS, E. Investigación del efecto del inmunomodulador homeopatico Modul8®, sobre sangre, lavado bronquial y ultraestructura de la fibrina en ratones experimentales asmáticos BALB/c. *Int. J. Morphol., 27(3)*:955-963, 2009.

RESUMEN: Modul8® es una mezcla compuesta de productos naturales que es conocida por ser un inmunomodulador. En el presente estudio, el efecto de este inmunomodulador se prueba de forma experimental en el modelo de ratón asmáticos BALB/c, para investigar sus propiedades sobre el conteo de glóbulos blancos en la sangre y lavado bronquial de los animales, ya que los glóbulos blancos desempeñan un papel fundamental en el proceso de respuesta inflamatoria implicado en el asma. Como es sabido, también las plaquetas desempeñan un papel importante en el sistema inmunológico, así, la ultraestructura de las plaquetas y las redes de fibrina también fueron investigadas por microscopía electrónica de barrido. Los animales fueron sensibilizados, nebulizados y tratados durante un período de 43 días hasta el término. Los resultados de los frotis de sangre, así como los de lavado bronquial revelaron un número significativamente mayor de eosinófilos en el grupo de asmáticos en comparación con el control y grupos tratados. Cambios en la ultraestructura de las plaquetas y redes de fibrina también pueden ser observados, donde el grupo tratado con la Modul8® aparece similar a el grupo control, donde los fibras de mayor grosor y menor grosor pueden ser claramente distinguidas y además, puede ser observada una apretada masa de plaquetas aglutinadas. Considerando las redes de la fibrina en animales asmáticos parecen endebles con una apretada masa de fibras de menor grosor que cubren las fibras de mayor grosor. Los agregados de plaquetas en asmáticos aparecen granulares sin el aspecto apretado del agregado plaquetario que rodea al grupo control. Por tanto, se concluye que Modul8® positivamente influye en el conteo de glóbulos blancos mediante la alteración del perfil de asmáticos a un aspecto similar al del control. Además, parece como si Modul8® tuviera un efecto estabilizador en las plaquetas y las redes de fibrina. De estos resultados se puede sugerir que Modul8® puede ser utilizado con éxito en el tratamiento de procesos inflamatorios como el asma.

PALABRAS CLAVE: Modul8®; Asma; Modelo de ratón BALB/c; Glóbulos blancos; Ultraestructura de plaquetas y fibrina.

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