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The Response of Vascular Dermal Endothelial Cells to House Dust Mite Extracts

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THE RESPONSE OF VASCULAR DERMAL ENDOTHELIAL CELLS TO
HOUSE DUST MITE EXTRACTS.

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science

By

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B.S., University of Akron, 2005

2008
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January 23, 2008

I hereby recommend that the thesis prepared under my supervision by Aaron Mathew Newman entitled The Response of Vascular Dermal Endothelial Cells to House Dust Mite Extracts be accepted in partial fulfillment of the requirements for the degree of Masters of Science.

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House dust mites are microscopic arthropods that can trigger moderate allergic symptoms such as sneezing, watery eyes, itching, and wheezing in sensitized individuals. People with more serious allergies to house dust mites can develop allergic diseases like atopic dermatitis and asthma. The effects of house dust mites on allergy sufferers make house dust mites and the study of their effects on the human body of great medical and economic importance.

A majority of the research that has been done on house dust mite’s effects on humans has dealt with the lungs and relevant disease like asthma. Little work has been done on the inflammatory response of the skin to the house dust mite. Since the human microvascular dermal endothelial cell (HMVEC-d) is a major regulator in the inflammatory response of the skin, my research attempted to look at the \textit{in vitro} effects of house dust mite extract on the expression of inflammatory molecules that are important in inflammatory cell trafficking and activation.

HMVEC-d were exposed to extracts of the two most common house dust mite species \textit{Dermatophagoides farinae} and \textit{Dermatophagoides pteronyssinus} at four different concentrations. The expression level of the cytokines IL-1\(\alpha\), IL-1\(\beta\), IL-6, macrophage
inflammatory protein 1α (MIP-1α), granulocyte-macrophage colony stimulating factor (GM-CSF), and eotaxin, as well as the chemokines IL-8 and macrophage chemoattractant protein 1 (MCP-1) were analyzed using a sandwich ELISA technique. The expression level of the chemokine receptors CXCR-1, CXCR-2, and CCR-5, and the adhesion molecules intracellular adhesion molecule 1 (ICAM-1), vascular cellular adhesion molecule 1 (VCAM-1), and E-selectin were analyzed using the indirect ELISA technique. Expression of all of the molecules was determined at 12 hours and 24 hours post exposure to house dust mite extract. The effects of house dust mite extract on dermal endothelial cells after stimulation with TNF-α was also tested.

The results showed an extensive upregulation in the expression of several of the molecules that were tested for. The adhesion molecule ICAM-1 had a significant dose dependent increase in expression but the adhesion molecules VCAM-1 and E-selectin showed a much smaller yet still significant dose dependent increase in expression. The cytokines and chemokines IL-6, GM-CSF, IL-8, and MCP-1 all showed a significant dose dependent increase in expression by the HMVEC-d. The chemokine receptors were unaffected by the extract and the cytokines IL-1α, IL-1β, MIP-1α, and eotaxin were not detected.

The results clearly show that the two species of house dust mite extracts caused an increase in the expression level of several specific inflammatory molecules by HMVEC-d. The variation in the effects that the two species of house dust mites had on HMVEC-d indicates that the uncharacterized non-allergic components of the two extracts may also play a role in the activation of the allergic response of the skin.
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House dust mites are very prevalent in homes in the United States and worldwide. They are the source of many allergenic proteins. About 27.5% of the general population in the United States has allergies to house dust mites (Arbes et al. 2005). House dust mites are microscopic arthropods that are related to spiders. They feed on human skin scales. Within a home mites are most abundant where skin scales collect such as mattresses, fabric covered furniture, and carpets. Their optimal growing conditions are 70-75% relative humidity and 20°C - 25°C (Arlian and Morgan 2003).

House dust mites are 70% water by weight and in order to maintain that hydration level they must absorb sufficient water vapor from unsaturated air to replace what they have lost by evaporation or bodily secretions. It is because of this delicate balancing act that house dust mites are so sensitive to the relative humidity of their environment. This is why house dust mites are prevalent in humid geographical areas of the world and not present in dry climates (Arlian and Veselica 1981, Arlian and Wharton 1974).

House dust mites can be cultured in the laboratory and aqueous extracts produced from them. These extracts contain water soluble molecules from the mite bodies and fecal material. Some of these molecules are allergens while some are not (Arlian et al. 1984). It is not known what effect the non-allergenic molecules from mites have on humans or their relationship to mite allergenic proteins. About 20 groups of allergens in these extracts have been isolated, named, and their allergenicity characterized. The individual mite allergens are assigned to these groups based on their biochemistry.

The group 1, 3, 6, and 9 are the protease allergens. Group 1 proteins are cysteine proteases and groups 3, 6, and 9 are serine proteases (Dilworth et al. 1991, King et al.
1996, Stewart et al. 1992, Yaseuda et al. 1993). These proteins have been shown to activate the components of the innate and adaptive immune system by several different mechanisms. They increase the paracellular permeability of the epithelial barrier by cleaving two of the main proteins found in the tight junctions (TJ) of lung epithelial cells (Wan et al. 1999, Wan et al. 2000, Sun et al. 2001).

These protease allergens have also demonstrated an ability to activate a receptor called protease-activated receptor (PAR) found on the surface of endothelial cells. Activation of the PAR causes a signal cascade that leads to a number of physiological effects, one being the release or altered expression of several different proinflammatory cytokines, adhesion molecules, chemokines, and their receptors (King et al. 1998, Sun et al. 2001, Asokananthan et al. 2002). The PAR also has been shown to have a direct effect on the tight junctions of endothelial cells (Carbajal et al. 2000, Kawkitinarong et al. 2004).

Most of the research that has been done on the effects of house dust mite extract on tight junctions and PARs has been done on epithelial cells of the respiratory system. Some research has been done on the effects of house dust mites on the skin but other cell types such as fibroblasts, keratinocytes and T cells (O’Brien et al. 1997, Nurse et al. 2000, Mascia et al. 2002) were the focus of those studies and not the microvascular endothelial cells that I used. The effect of non-allergenic molecules from mites on the immune and inflammatory effector cells has not been determined.

I examined the proinflammatory cytokines and other molecules expressed by endothelial cells of the skin following exposure to house dust mite extract. We want to compare the reaction of endothelial cells of the skin to house dust mite extract with the...
reaction to *Sarcoptes scabiei* extract by endothelial cells of the skin. This comparison of the reactions between these two closely related mites could prove to be very beneficial to understanding the mechanics behind the two reactions.

**Specific Aim**

My specific aim for this project was to test the hypothesis that certain molecules in house dust mite extract will either up regulate, down regulate, or not affect the expression level of the cytokines, adhesion molecules, chemokines, and the chemokine receptors tested for in this project by dermal endothelial cells.
**Background**

**General House Dust Mite Importance**

Allergenic house dust mites (HDM) are microscopic arthropods that feed on human skin scales. They are found in bedding material, carpets, drapes, and upholstered furniture in human dwellings where skin scales accumulate. The most important species are *Dermatophagoides pteronyssinus, D. farinae,* and *Euroglyphus maynei* (Arlian and Morgan 2003). These are the species of mites that are most commonly found in house dust (Arlian 1989). It was even said by Voorhorst et al. in 1964 that the mites of the genus *Dermatophagoides* were the most important single source of house dust allergens.

House dust mites and the allergens they produce are very prevalent in homes and a large portion of the population is genetically predisposed to allergies two factors that combine to make allergies to house dust mites very common.

Allergies to house dust mites are very prevalent in the United States. The third National Health and Nutrition Examination Survey (N.H.A.N.E.S III) was a national survey of the prevalence of people allergic to ten common allergens (dust mite, German cockroach, cat, perennial rye, short ragweed, bermuda grass, Russian thistle, White oak, *Alternaria alternate,* and Peanuts) (Arbes et al. 2005). Out of the 10,508 people tested using the skin-prick method, 43.0% were allergic to at least one of the 10 allergens tested. The study also showed that among those people tested, 27.5% had positive skin tests to house dust mites compared to 26.1% for German Cockroaches, and 17.0% for cats. Six of the allergens that were tested for in the N.H.A.N.E.S II study as well as in the N.H.A.N.E.S III study, showed a 2.1 to 2.5 percent increase in prevalence of allergies to
those allergens in the past 20 years since the H.N.A.N.E.S II was conducted and published in 1987 (Gergen et al. 1987).

Allergies to house dust mites are not only found in the United States. They are a global health problem. Several studies have been conducted that have quantified the prevalence of different species of house dust mites in dwellings and the prevalence of house dust mite allergy across the globe in such places as Latin America (Fernandez-Caldas et al. 1993), Reykjavik, Iceland (Hallas et al. 2004), Pavia, Italy (Moscato et al. 2000), Porto, Portugal (Placido et al. 1996), and Australia (Tovey et al. 2000).

**Classification of house dust and related mites**

House dust mites are placed in the phylum *Arthropoda* which means jointed appendages and the subphylum *Cheliceriformes* which consists of organisms with chelated mouthparts. In contrast, insects and centipedes are placed in the subphylum *Uniramia* and thus mites are not very closely related to them, with many different morphological and physiological differences between the two groups (Arlian and Platts-Mills 2001). Mites are arachnids and thus more closely related to spiders and scorpions. Household dust mites are placed in the subclass *Acari* along with mites and ticks. The members of *Arachnida* have eight legs, chelated mouthparts, and no antennae.

Within the subclass *Acari*, house dust mites are placed in the suborder *Astigmata*. The suborder *Astigmata* gets its name from the fact that about 5,000 of its identified species lack stigmata (openings in their body for the purpose of gas exchange). The suborder *Astigmata* also includes two other important types of mites: scabies mites and
stored product mites also called storage mites. In general, storage mites are usually found in stores of grain, hay, and straw and the human and animal products made from these items (Cuthbert et al. 1979, Jeffery 1976, Terho 1982). Like house dust mites, storage mites are also a source of many potent allergens. More sensitization to storage mites occurs in occupational settings than in the urban setting, unlike with house dust mites which are more prevalent in homes (Cuthbert et al. 1979). An example of sensitization to storage mites in rural occupational settings is shown in a study done by van Hage-Hamsten et al. (1985), which showed 12% of 440 farmers from Gotland, an island in the Baltic Sea, had allergies to one or more storage mites. Other studies also show a significant relationship between people in an occupational setting and the presence of storage mite allergies (Jeffery 1976, Terho et al. 1982, Revsbech and Andersen 1987, Revsbech and Dueholm 1990, Marx et al. 1993).

Some of the more common storage mite species found in the occupational setting are *Leidoglyphus destructor*, *Acarus siro*, *Tyrophagus putrescentiae*, and *Glycyphagus domesticus* (van Hage-Hamsten et al. 1985). *Blomia tropicalis*, a storage mite from the family *Echimyopodidae*, is unique because unlike most of the other storage mite species this one is often found in homes located in tropical and semi-tropical climates that possess the proper conditions (Arlian and Morgan 2003).

The scabies mite, *Sarcoptes saciei*, is an ectoparasite of humans and many other mammals. This species is related to the house dust and storage mites and it is immunogenetically highly cross-reactive with house dust mites (Arlian et al. 1988, Arlian et al. 1991, Arlian et al. 1995).
Morphology

Mites are too small to be seen with the unaided eye. Females of the *D. farinae* species usually have a fresh weight of 10.7±2.3 µg (Arlian and Wharton 1974) and range in body length from 395-435 µm, their width is 285-305 µm (Fain 1967). The males are much smaller, with a fresh weight of 4.1±1.0 µg and range in body length from 291-324 µm and their width is 210-231 µm. There is a similar size difference between the males and females of the *D. pteronyssinus* species (Fain 1966). Females have a fresh weight of 5.8±0.2 µg (Arlian 1975) and range in body length from 305-360 µm and have a body width of 201-255 µm. The males of the *D. pteronyssinus* species have a fresh weight of 3.5±0.2 µg (Arlian 1975) and range in body length from 240-290 µm and have a body width of 180-192 µm.

Male dust mites exhibit anal suckers, external dorsal sclerites (shields) on the posterior body basal apodemes of the genitalia, coxal I apodemes, varied leg size and chaetaxie. Female dust mites exhibit specific genital apertures and the associated apodemes and a copulatory bursa complex. These features allow easy identification of male versus female dust mites and the different species (Arlian 1975, Fain 1965, Fain 1966, Fain 1967, Griffiths and Cunnington 1971, Wharton 1976).

Chelicerae and the pedipalps extend from the anterior of the dust mites body. Together they are called the gnathosoma. Dust mites use the chelicerae, which are pincher like appendages, to hold food or, in the case of predacious mites, capture food. The pedipalps are sensory structures used to taste their food. Their food is liquefied in the preoral area. The viscous solution of food and saliva that is produced is then sucked through the oral opening (Walter 1999).
Posterior to the gnathosoma is the mite’s body that is called the idiosoma. The organs of digestion, excretion, reproduction and the brain are all located in the idiosoma. This interaction of the head, abdomen, and thorax into one soma or body is uncommon among arthropods, which usually have a segmented soma. The idiosoma of the mite is encased in an exoskeleton. The exoskeleton is covered in a waxy coating that prevents water loss to the outside environment.

Four pairs of legs extend from the idiosoma. The most anterior pair of legs (legs I) are used by the mite to sense the size of food, examine a potential mate, and to check the stability of the substrate in front of them. In some predatory mites, legs I are often used in conjunction with the palps and the chelicerae to form a basket like structure that is used to trap their prey (Linquist and Walter 1989). Both the pedipalps and the first tarsus of legs I have several clusters of sensory setae situated on them (Griffiths and Cunnington 1971). The last three pairs of legs (legs II, III, and IV) are used for locomotion, but they also have many chemical and mechanical sensory receptors on them as well.

Organisms from the suborder Astigmata lack an organized respiratory system; thus they have no openings on the surface of their body for the purpose of gas exchange (Arlian and Morgan 2003). They still require oxygen for aerobic respiration and produce carbon dioxide.

House dust mites are about 70% water by weight and must obtain water from the humid ambient air in their environment in order to balance what they have lost due to evaporation and bodily processes (Arlian and Veselica 1981, Arlian and Wharton 1974). House dust mite and storage mites can live in environments where there is no liquid water. These mites are able to do this because they can absorb most of the water they
need from humid ambient air if the relative humidity is sufficiently high. The importance of ambient relative humidity is discussed later.

**Ecology**

**Life Cycle**

There are a total of five sequential developmental stages in the life cycle of house dust mites. They consist of egg, larva, protonymph, tritonymph, and adult (male or female). Once a larvae emerges from an egg it actively feeds. After feeding the larvae becomes quiescent or inactive and metamorphosis to the next stage in the life cycle takes place. The process is repeated until an adult is formed (Arlian et al. 1990, Arlian and Dippold 1996).

House dust mites exhibit external morphological features that are unique to each stage of their life cycle (Arlian and Morgan 2003). This change in morphology allows for the identification of the individual stages in the house dust mite life cycle. The larvae are hexapodal and lack ventral and genital setae, genital papillae, and some dorsal and lateral body setae. Once in the protonymph stage the mite gains a set of legs and becomes octopodal. It also gains one pair of genital papillae, and all anal, genital, dorsal, and lateral setae become present. The last stage before adulthood, the tritonymphal stage, the mite gains two pairs of genital papillae between coxae IV, but still has no genital opening. During the adult stage the mite gains a genital opening between legs III for the female and between legs III and IV for the male. They also gain two pair of genital papillae between legs III and IV. Bursa copulatrix, duct and seminal receptacle complex
develop in females. Males that enter the adult stage develop anal suckers, a penis, and associated sclerotized structures (Wharton 1976).

Temperature and Relative Humidity’s Affect on Mites

The relative humidity of the mite’s environment has a large effect on the length of life cycle and the individual stages of the life cycle as well as the overall survivorship of a house dust mite population (Arlian et al. 1998, Arlian et al. 1990, Arlian et al. 1999). In *D. farinae* species low relative humidity increases the duration of the mite’s immature stages (Arlian et al. 1999). The dependence on relative humidity is due to the fact that dust mites can only replace the water that they loose due to transpiration through the body and secretion of bodily fluids, by absorbing water vapor from unsaturated air. To be able to absorb the water needed the relative humidity has to be at or above a critical minimum level also called the critical equilibrium humidity (CEH) (Larson et al. 1969). For the dust mite *D. farinae* the CEH ranges from 52% to 73% depending on temperature (Arlian and Veselica 1981). The actual water absorption in dust mites is accomplished by their releasing a hyperosmotic solution from the superacoxal glands that open above the first pair of legs (Arlian 1992). The solution absorbs water as it flows into the preoral cavity where the hydrated fluid is then ingested (Wharton and Furumizo 1977).

House dust mite’s optimal conditions for development are 20°C to 25°C and 70% to 75% relative humidity (Arlian et al. 1990, Arlian and Dippold 1996). When the temperature or the relative humidity is increased, development time shortens. If the temperature and relative humidity decrease then the mite’s development time lengthens (Arlian et al. 1999). The following experiments have demonstrated this relationship
(Arlian et al. 1990, Arlian and Dippold 1996). At a temperature of 16°C and a relative humidity of 75%, both *D. pteronyssinus* and *D. farinae*, male and female, show a duration of their life cycle (egg to adult) to be 122.8±14.8 days and 140.1±14.7 days, respectively. The length of the life cycle for *D. pteronyssinus* at 35°C and 75% relative humidity is 15.0±2.0 days. The length of the life cycle for females of *D. farinae* at 35°C and 75% relative humidity is 22.1±3 days. *D. farinae* is not able to develop from the egg stage to the adult stage at temperatures of 16° and 35°C, whereas *D. pteronyssinus* could complete development.

House dust mite fecundity is also greatly affected by temperature (Arlian and Dippold 1996, Furumizo 1975). *D. farinae* grown at 21.1°C had a mean oviposition period (egg laying period) of 27.6 days. In that period they laid on average of 42.0 eggs per female. When the mite was incubated at 26.6°C, the mean oviposition period was 19.8 days, with a total mean number of eggs laid of 50.3 per female mite. The fecundity of female *D. farinae* at 32.2°C differed quite a bit from the previous incubation temperatures. The mean oviposition period was 12.2 days. Each mite only produced on average of 28.2 eggs during this egg laying period (Furumizo 1975).

Temperature and relative humidity can also affect population growth rates of house dust mites. At 20°C and 65% to 75% relative humidity *D. pteronyssinus* has a population growth rate of 30% to 35% per week, while *D. farinae*’s population growth rate is 16% to 19% per week (Arlian et al. 1998). Because of the relationship between prevalence of house dust mites and the climatic conditions, manipulations of their microenvironment can be used to reduce house dust mite levels in homes. Keeping the relative humidity in a house below 50% coupled with weekly vacuuming and cleaning is
recommended to decrease house dust mites and allergen levels in homes (Arlian et al. 2001).

Seasonal fluctuations in temperature and relative humidity causes dust mite populations to grow or decline accordingly (Arlian et al. 1983). In temperate climates, live dust mite populations are highest during the humid summer months (May, June, July, and August). Population levels usually reach their maximal levels in the later summer months, (July and August). In the study conducted by Arlian et al. (1983) between 1977 and 1979 the average humidity indoors during the summer months was between 70% and 75% which is the ideal relative humidity for dust mites to thrive. Mite populations declined during the fall and bottomed out during the dry winter months (December thru April). The average relative humidity during the winter months of the study was about 35% to 45%, which is below the critical minimum humidity needed for mite survival.

A dust mite population in a home is not completely killed off during the dry winter months because mites, when threatened with dehydration, will develop into a quiescent desiccation-resistant protonymph stage in its life cycle (Arlian et al. 1983). Being in the pharate tritonymph stage allows the mite to survive longer in the low relative humidity conditions of the winter months. So ultimately it is the indoor relative humidity cycle that is affected by the seasonal outdoor temperature that in turn affects the dust mite population.
Geographical Prevalence

Temperature and relative humidity not only affects house dust mite development but they also dictate where they are usually found in a particular home as well as where they can be found geographically. In a study by Arlian et al. (1992), medically significant quantities of house dust mites, meaning a dust sample contained at least 100 mites/gm of dust (Korsgaard 1983), were found in a number of humid geographical areas in the United States such as Cincinnati, OH., New Orleans, LA., and Memphis, TN., Delray Beach, FL., and Los Angeles, CA. The results of the study showed that the quantity of house dust mites varied among the different homes within each geographic location. The study also showed that the overall averages of dust mite densities in the different cities varied a great deal as well. Homes in Cincinnati and New Orleans had higher numbers than the homes in Delray Beach and Los Angeles.

Another interesting observation made by the authors of this study was that most of the homes (>74% of all the homes tested) were coinhabited by both *D. pteronyssinus* and *D. farinae* species of mites. In these coinhabited homes there was a dominant species which made up to 75% of the total mite population of the home. There was no trend as to what species was dominant (Arlian et al. 1992).

Another study shows a variation in the prevalence of a mite population within the different areas of a home (Arlian et al. 1982). Mites are most prevalent in areas of a home where there is the most foot traffic, since that is where human skin scales, their food source, are in highest concentration. There are other factors that affect the house dust mite concentration in the different areas of the home (mattresses, bedroom floors, family room floors, and family room furniture) as well, such as temperature, relative humidity,
and physical conditions (fabric, wood, or metal). In the Arlian 1982 study, mattresses showed the lowest amount of dust mites compared to the other sites tested. The highest mite levels were seen on the carpeted bedroom floors.

Immunological Effects

The Allergens

The house dust mite is the source of many potent allergens. These allergens come from many different parts of the mite body. Many of these allergens have been isolated and characterized (Table 1). These allergens are named and classified into several groups according to chronology of discovery and biochemical characteristics (Table 1). The proper nomenclature of allergens is the first three letters of the genus, a space and then the first letter of the species name. After that there is another space and then a number which stands for the order of discovery (King et al. 1994).

Group 1 proteins have two globular domains formed independently by the N and C terminal sequences (Dilworth et al. 1991). The cleft between the two domains is where substrate binding and the catalytic residues reside. There is a long outside flexible loop structure that connects the two domains. The sequence of the loop structure was found to be the most allergenic part of the group 1 proteins (Hales and Thomas 1997). In an extract of \textit{D. pteronyssinus}, the group one allergen was shown to be the most reactive allergen in the extract, binding upwards of 75% of the total IgE antibodies in allergic patient sera that were specific for \textit{D. pteronyssinus} (Chapman and Platts-Mills 1978, Platts-Mills and Chapman 1987). More than 90% of dust mite sensitive patients tested
reacted to group 1 allergens which demonstrates the overall allergenicity of the group 1 proteins.

Group 2 allergens show about 88% sequence homology between *D. farinae* and *D. pteronyssinus* (Stewart 1995). The cDNA sequence studies of the group 2 allergens showed some polymorphisms (Yuuki et al. 1990), but unlike *Der p 2* the group 1 allergen *Der p 1* was observed as having more sporadic change in its polymorphisms than *Der p 2* did (Chua et al. 1996).

The group 3 proteins were found to be trypsin like serine proteases based on substrate specificity and sequence homology studies. The proteins *Der p 3* and *Der f 3* are homologues with about 85% homology between the two. The trypsin protein *Der p 3* showed 100% reactivity in selected sera from mite allergic donors, which shows that it is one of the major allergens in a whole mite extract (Stewart et al. 1992).
Table 1. Mite Allergens. List of mite allergens their names, their molecular weight, their frequency of reactivity, and their homology to known proteins.

<table>
<thead>
<tr>
<th>Allergen Group</th>
<th>Specific Allergens Characterized</th>
<th>M.W. (kDa)</th>
<th>Frequency of Reactivity (%)*</th>
<th>Homology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Der f 1, Der p 1, Eur m 1, Blo t 1</td>
<td>25-39</td>
<td>&gt;90 (8,12,22)</td>
<td>Cysteine protease, homology similar to enzymes papain, actinidin, cathepsin H &amp; B, bromelain, frein</td>
</tr>
<tr>
<td>Group 2</td>
<td>Der f 2, Der p 2, Eur m 2, Tyr p 2, Lep d 2, Gly d 2</td>
<td>14</td>
<td>&gt;90 (8,12,23)</td>
<td>Homology with primate epididymal or insect molting protein</td>
</tr>
<tr>
<td>Group 3</td>
<td>Der f 3, Der p 3, Eur m 3, Blo t 3</td>
<td>23.8-30</td>
<td>57-90 (12,13,24)</td>
<td>Trypsin-like serine protease</td>
</tr>
<tr>
<td>Group 4</td>
<td>Der f 4, Der p 4, Eur m 4</td>
<td>56-63</td>
<td>25-46 (5,32)</td>
<td>Amylase</td>
</tr>
<tr>
<td>Group 5</td>
<td>Der p 5, Blo t 5, Lep d 5</td>
<td>14-15</td>
<td>45-60 (6,14,25)</td>
<td>Unknown</td>
</tr>
<tr>
<td>Group 6</td>
<td>Der f 6, Der p 6</td>
<td>25</td>
<td>40-60 (10)</td>
<td>Chymotrypsin-like serine protease</td>
</tr>
<tr>
<td>Group 7</td>
<td>Der f 7, Der p 7, Lep d 7</td>
<td>22-28</td>
<td>50 (11,25)</td>
<td>Unknown</td>
</tr>
<tr>
<td>Group 8</td>
<td>Der p 8</td>
<td>25-26</td>
<td>40 (7)</td>
<td>Rat &amp; Mouse glutathione-S-transferase</td>
</tr>
<tr>
<td>Group 9</td>
<td>Der f 9, Der p 9</td>
<td>24-28</td>
<td>80 (4)</td>
<td>Collagenolytic serine protease</td>
</tr>
<tr>
<td>Group 10</td>
<td>Der p 10, Blo t 10, Der f 10, Lep d 10</td>
<td>33-37</td>
<td>&gt;60[Lep13%] (1,26,27)</td>
<td>Melanogaster, rabbit &amp; human alfa-tropomysin</td>
</tr>
<tr>
<td>Group 11</td>
<td>Der f 11, Blo t 11, Der p 11</td>
<td>98-110</td>
<td>High (15,28,29)</td>
<td>Paramyosin</td>
</tr>
<tr>
<td>Group 12</td>
<td>Blo t 12</td>
<td>14</td>
<td>50 (9)</td>
<td>Unknown</td>
</tr>
<tr>
<td>Group 13</td>
<td>Blo t 13, Aca s 13, Lep d 13, Tyr p 13</td>
<td>15-17</td>
<td>10 (2,25,30,31)</td>
<td>Cytosolic fatty acid-binding proteins</td>
</tr>
<tr>
<td>Group 14</td>
<td>Der f 14, Der p 14, Eur m 14</td>
<td>177-190</td>
<td>70 (3)</td>
<td>Apolipophorin-like protein</td>
</tr>
<tr>
<td>Group 15</td>
<td>Der f 15</td>
<td>98/109</td>
<td>70 (16)</td>
<td>Chitinases insect exoskeleton component (found in digestive tract not in fecal pellets)</td>
</tr>
<tr>
<td>Group 17</td>
<td>Der f 17</td>
<td>30</td>
<td>35 (18)</td>
<td>Calcium-binding protein</td>
</tr>
<tr>
<td>Group 18</td>
<td>Der f 18</td>
<td>60</td>
<td>57-77 (19)</td>
<td>Chitinases insect exoskeleton component (found in digestive tract not in fecal pellets)</td>
</tr>
<tr>
<td>Group 19</td>
<td>Blo t 19</td>
<td>7</td>
<td>10</td>
<td>Antimicrobial peptide</td>
</tr>
<tr>
<td>Group 20</td>
<td>Der f 20</td>
<td>15</td>
<td>9.8 (21)</td>
<td>Heat shock protein 70 family</td>
</tr>
</tbody>
</table>

*References given in parentheses.

†Table from (Arlian 2002).
The groups 1, 3, 6, and 9 are all serine or cysteine proteases. It has been suggested that the proteolytic activity of these proteins affects their allergenicity (Thomas et al. 2002). The protease proteins from house dust mites elicit an allergic reaction by having antigenic determinants that are recognized by IgE but they can also produce immune effects in other ways. One study by Maruo et al. (1997) showed that the group 3 allergens were able to activate the complement components C3 and C5 by proteolytic cleavage to produce C3a and C5a, respectively. The peptides C3a and C5a are anaphylatoxins used in the complement system to combat infection and also play a role in the inflammatory response. The anaphylatoxins may also have a role in the pathogenesis of a number of different allergic diseases (Maruo et al. 1997).

The protease allergens, groups 1, 3, and 9, can stimulate an immune reaction, allergic or not, by activating certain receptors on lung epithelial cells. These receptors in turn signal the production of proinflammatory cytokines such as eotaxin and GM-CSF (Sun et al. 2001).

Inflammation/Influence of House Dust Mites on Tight Junctions.

If house dust mite allergens are inhaled, one of the first points of contact that they have with the body is the mucous membrane of the nose and at the epithelial barrier of the lungs. When the protease allergens make contact with the epithelium some of the allergens bind to and activate PARs (Protease Activated Receptor) found on the surface of the bronchial epithelial cells. This activation of the PARs leads to the expression of several proinflammatory molecules (Asokanathan et al. 2002, Sun et al. 2001). Some of the protease allergens make their way to the tight junctions (TJ) of the epithelial barrier
where they cleave the extracellular exposed portions of the proteins occludin and claudin, proteins essential in the proper functioning of the TJ, this cleavage causes an increase in the epithelial barrier’s permeability. This increase in permeability allows the protease allergens to enter the tissue where they encounter APCs and other inflammatory cells. The cytokine cascade and the allergens encounter with APCs and other inflammatory cells begins the inflammatory response and subsequent adaptive immune response.

PAR’s are a family of seven transmembrane G protein-coupled cell surface receptors. Serine proteases activate them by cleaving a specific site on the receptor’s extracellular N-terminus. The newly exposed N-terminus acts as a tethered ligand and binds to another site on the receptor which activates it. The activation processes is irreversible and once it is cleaved the whole receptor is degraded in the lysosome and a new receptor is made and is transported to the plasma membrane (Reed and Kita 2004).

So far there are four documented PAR receptors designated PAR 1-4 (Vu et al. 1991, Nystedt et al. 1995, Ishihara et al. 1997, Xu et al. 1998). The expression of a number of different proinflammatory cytokines and chemokines are regulated by the different PAR’s. For example Der p 1 and Der p 9 both increase expression of eotaxin, and GM-CSF upon exposure to PAR-2 on cells of the airway epithelium (Sun et al. 2001). Der p 1 was also shown to increase expression of IL-6 and IL-8 through the activation of PAR-2 (Asokanathan et al. 2002).

In addition to activating PAR’s the proteolytic allergens of house dust mites also have a large effect on TJ of epithelial cells (Herbert et al. 1995, Wan et al. 1999, Wan et al. 2000). The tight junction’s purpose is to create a semi leak proof seal between the
apical and basolateral membrane areas of the epithelial and endothelial barriers (Wu et al. 2000).

The TJ complex is made up of strands of protein embedded in the plasma membrane. Once these strands encounter a strand on another cell they become fused into a tight junction complex (Tsukita and Furuse 2002). The TJ complex is made up of several different proteins. The zonula occludens (ZO-1 and ZO-2) are peripheral proteins that form the anchorage points for the transmembrane proteins. Occludins, claudins, and junctional adhesion molecules (JAM) are the integral transmembrane proteins that form the strand or bridges between the ZO-1 anchors on the extracellular surface of the plasma membrane (Wu et al. 2000).

The occludin protein is a major protein in the TJ complex. It consists of two extracellular loops, one smaller intracellular loop, four transmembrane regions, a cytoplasmic N-terminal region, and a cytoplasmic C-terminal region. The ZO-1 anchor binds to the C-terminal region of occludin (Harhaj and Antonetti 2004).

Protease allergens like Der p 1 have the ability to cleave the proteins in the TJs between the epithelial cells lining the lung’s airways (Wan et al. 1999, Wan et al. 2000, Sun et al. 2001). The allergen causes the TJ to break apart by cleaving the protein occludin at the first external loop segment after which the intracellular protein ZO-1 somehow becomes exposed to the proteolytic activity of the protease (Wan et al. 2000). If the two proteins occludin and ZO-1 fail to function properly due to them being cleaved, the whole TJ complex will break up allowing the epithelial cells to become much more permeable.
When the endothelial barrier is functioning normally it prevents foreign particles from entering the bloodstream. If the structural integrity of the barrier decreases because of the destruction of the TJ proteins the permeability will increase, allowing foreign particles such as allergens, access to dendritic antigen-presenting cells via the paracellular pathway (Wan et al. 1999).

On an interesting note there have been several studies done that show a close relationship between PAR activation and tight junction permeability (Carbajal et al. 2000, Kawkitinarong et al. 2004). Thrombin, a serine protease, like the house dust mite allergens from groups 3, 6, and 9, is important in blood coagulation. Thrombin is able to cleave and activate PARs 1, 3, and 4. After the thrombin induced activation of a PAR, a very complex signal cascade takes place leading to a number of cellular effects such as chemotaxis, proliferation, release of inflammatory molecules, as well as an increase in vascular endothelial cell barrier permeability.

Immune Response to House Dust Mites.

Once the house dust mite allergens are internalized by antigen presenting cells (APC) of a genetically susceptible person, the APCs activate helper T-cells (Th0). The activated helper T-cells differentiate into Th2 cells and go on to initiate a Th2 response. The Th2 cells also present the antigen and activate B-cells. The B-cells internalize the allergen and display it in association with a MHC-II molecule. Th2 cells interact with this B-cell at the MHC-II complex or B-cell receptor, and produce cytokines that stimulate proliferation of the B-cell and its differentiation to an IgE producing plasma cell. This production of IgE is the hallmark of an allergic reaction (Cohen 1988).
specific IgE antibodies become bound to the Fc receptors on the surface of mast cells and basophils. Once this occurs the individual is said to be sensitized. These mast cells take up residence in tissue such as the dermis of the skin, mucous membranes of the nose and eyes and in the lungs.

If the sensitized individual encounters that allergen again the F(\text{ab}')_{2} portion of the IgE antibody that is already bound to the mast cell or basophil will bind to the allergen. The cross-link binding of multiple IgE’s to the epitopes on the allergen bound to the receptors on the mast cells causes the Fc receptors on the surface of those cells to move together which triggers the mast cell to release chemical mediators such as histamine, prostaglandins, and leukotrienes. These chemical mediators can cause vasodilatation, itching, constriction of smooth muscle, and changes in membrane permeability. All these effects lead to the production of the clinical symptoms that are most commonly seen in an allergic reaction (Arlian 2002). All together this response, is termed type I hypersensitivity reaction.

The molecules tested for in this research were chosen because of their importance in the inflammatory response. The adhesion molecules are important for extravasation the process of bringing white blood cells from the blood stream into the tissue were the inflammation is taking place. The chemokines IL-8 and MCP-1 are also important in the extravasation process because they attract the white blood cells to the endothelium in the first place. The cytokine IL-6 has a number of effects during the early stages of the inflammatory response one important one is it acts as a amplifying molecule by signaling cells to express more and more IL-6. The cytokine GM-CSF comes to play later in the
inflammatory response by signaling the maturation of tissue macrophages and other granulocytes.

The type of allergic disease that results depends on the path of exposure to the allergen. Clinical symptoms can include nasal rhinitis (allergen entering the upper airway and causing itching and runny nose), conjunctivitis (allergen entering the eye and causing itching and watery eyes), systemic anaphylaxis e.g. bee sting (allergen entering the bloodstream), and atopic dermatitis (allergen being exposed to the skin) (Casolaro et al. 1996).

Allergic reactions in the skin and in the lungs are similar in a lot of ways. They both produce IgE hypersensitivity responses, they both produce inflammatory reactions, and they both manifest clinical symptoms. The difference comes in the details. In order for allergens to gain access to the dermis where the inflammatory responses begins they need to get through the epidermis which consists of an inner layer of stratified squamous epithelial cells and an outer layer of non mitotic highly keratinized epithelial cells. Once the allergens land on the skin the individual will scratch allowing the allergens access to the inner layer of the epidermis. The allergens then encounter the stratified squamous layer of epithelial cells which are bound to each other by tight junctions located at regular intervals along the lateral domain of the epithelial cells. The ability of protease allergens to break apart the TJ is what allows the allergen access to the dermis. From there the allergens encounter most of the same inflammatory effector cells that they would encounter in the lungs. Exposure of allergens to the lungs can lead to asthma whereas exposure of allergens to the skin can lead to atopic dermatitis.

Atopic dermatitis, triggered by house dust mites, is an important disorder that is not well understood. Dermal endothelial cells have been found to be a major player in the
regulation of extravasation of immune and inflammatory effector cells in an acute or chronic inflammatory response with somebody that suffers from atopic dermatitis (Steinhoff et al. 2006). Understanding how dermal endothelial cells regulate the inflammatory and immune responses to specific biological agents in an atopic individual could lead to a better understanding of atopic dermatitis.

The goal of this research is to understand the regulatory role of endothelial cells in the overall allergic response. Endothelial cells have already been shown to have a large effect on the outcome of exposure to an allergen and other bioactive molecules. A more specific goal of this research was to determine how skin endothelial cells respond to house dust mite products. We studied the expression of different proinflammatory cytokines, chemokines, and their receptors as well as certain adhesion molecules by human dermal endothelial cells after exposure to house dust mites and their allergens.
Materials and Methods

Overview of Protocol

Cultured vascular dermal endothelial cells were exposed to extracts of house dust mites, TNF-α, or both house dust mites and TNF-α for a 12 hour and 24 hour period of time. TNF-α is a proinflammatory cytokine that induces an immune response by most cell types when they are exposed to it. The stimulatory cytokine TNF-α was used in these experiments in order to see if the house dust mite extract was able to down regulate cytokines that was up regulated by TNF-α. The supernatants were removed from the three sets of cells and assayed for several pro-inflammatory cytokines and chemokines such as IL-6, IL-8, IL-1α, IL-1β, MCP-1, MIP-1α, eotaxin, and GM-CSF that were secreted by the cells. Cells cultured in medium without any stimulation served as controls to determine constitutive expression. In addition, the cells were assayed for expression of the cell surface adhesion molecules ICAM-1, VCAM-1, and E-selectin, and the cell surface chemokine receptors CXCR-1, CXCR-2, and CCR-5.

House Dust Mite Extract

Live D. pteronyssinus and D. farinae dust mites were collected by brushing them from the lids of their culture jars. This was done by employees in the lab. The collected mites were stored at -20º C and were brought to room temperature when needed. Mite extracts were prepared as previously described (Arlian et al. 1984). Half a gram each of the house dust mite cultures was added individually to a 15ml TenBroeck homogenizer
along with 5ml of glass distilled water for a 1/10 (wt:vol) dilution. Some experiments were ran using a second extract that was at a 1/5 (wt: vol) dilution. The homogenizers were covered with parafilm and allowed to extract at 4º-8ºC for 24-48 hours. The homogenizers were removed from the refrigerator and the liquid inside was homogenized by screwing with the pestle for 10 screws or until smooth. The resulting liquid was poured into a 15ml falcon tube and centrifuged for 15 minutes at 2700rpm (1173g). A 5ml syringe was attached to a 0.22um sterile filter. The supernatant was removed and put into the syringe. The extract was injected through the filter and into a sterile vial. Some of the extract was injected into a microfuge tube and both were stored at 4º-8ºC until needed.

A Bradford protein assay was performed (Bradford 1976) on the extract in the microfuge tube. A series of doubling dilutions with bovine serum albumin (BSA; Fisher Scientific Pittsburg, PA) and glass distilled water with protein assay dye (Bio-Rad, Hercules, CA) added was made and rub in a Spectronic 601 spectrophotometer (Spectronic Instruments, Inc.; Rochester, NY). The absorbencies of the house dust mite extract was compared to the standard curve and the protein concentration was calculated.

**Cell Culture**

Human adult dermal microvascular endothelial cells (HMVEC-d), purchased from Cambrex Bio Science Walkersville, Inc. (Wakersville, MD), were used for these studies. The cryopreserved cells were thawed in endothelial cell basal media-2 with growth supplements (hEGF, hydrocortisone, GA-1000, FBS, VEGF, hFGF-B, R³-IGF-1,
and ascorbic acid). The cells were incubated in a 37°C incubator with 5%-6% CO₂. The cells were allowed to incubate until they reached a 70%-80% confluency determined visually. The cells were then washed with EBM-2 and trypsin/EDTA was then added to release the cells from the cell culture flask. Once most of the cells were rounded and floating the trypsin/EDTA was neutralized with 1% FBS/EBM-2. The cells were removed from the flask and centrifuged for 10min. at 1400 RPM (140g). The cells were resuspended in EBM-2 and counted. After being counted the cells were plated in Costar 96 well culture plates (Corning Glassworks, Corning, NY) at 4,000-14,000 cells per well along with 200ul of media. The cells were counted using a hemocytometer. The cells were averaged and then multiplied by the dilution factor of the hemocytometer (10^4) which gave the total number of cells in the flask. Once that number was multiplied by the amount of liquid the cells were suspended in each well of the culture plates, the cells per well was calculated.

**Cell-based ELISA**

The cells in the Costar 96 well culture plates were incubated for 72 hours. After incubation the plates were inspected visually and found to have reached a confluency of between 70% and 80% in each well. After the visual inspection 100ul of media was removed from each well. A premixed solution of house dust mite extract and EGM-2 media was added to each well in varying concentrations. The concentrations used were 200ug/ml (150ug/ml used for the third run), 100ug/ml, and 25ug/ml. The TNF-α stimulated experiments required the addition of TNF-α at a 4ng/ml concentration to their
extract mixtures. These sets of experiments allowed us to see what effect house dust mite extract had on endothelial cells that were already immunologically stimulated. The two plates were allowed to incubate for a 12 hour and a 24 hour period. Once the incubation period was over, 150ul of the media was removed from each well and combined with the media from wells that were exposed to the same concentration of extract and the same species of mite in a microfuge tube. The microfuge tubes were centrifuged for 10 minutes at 8,765 g to pellet any remaining cellular debris. The supernatants were collected into bullet tubes and then stored at -80°C until they were assayed for cytokines. The 96-well plates were then washed with a Dulbecco’s phosphate buffered saline with 0.05% Tween-20 washing solution (PBST; Sigma-Aldrich, St. Louis, MO.). The plates were then blocked with a 2% EBM-2/BSA solution and incubated in a 37°C incubator with 5%-6% CO₂ for half an hour. The plates were washed twice with PBST and 1:500 dilutions of the primary antibodies which were monoclonal biotinylated antibodies directed against ICAM-1 and VCAM-1 both from eBioscience, Inc. (San Diego, CA) along with monoclonal biotinylated antibodies against E-selectin were purchased from R&D Systems, Inc. (Minneapolis, MN) were added at a 50ul volume and allowed to incubate for a half an hour. In the ELISAs for the chemokine receptors CXCR-1, CXCR-2, and CCR-5 the primary antibodies that were used were monoclonal non-biotinlyated antibodies all from R&D Systems, Inc. The primary non-biotinlyated antibody was used due to the lack of availability of the monoclonal biotinylated antibodies that were used against the adhesion molecules. The plates were washed twice with PBST and 50ul of a 1:2000 dilution of the secondary antibody which was strepavidin conjugated with horseradish peroxidase was used for the biotinylated antibodies. In the chemokines,
receptor experiments a goat-anti mouse Ig antibody was used for the non-biotinylated antibodies. Both secondary antibodies were added to each well and allowed to incubate for another half an hour. The secondary antibody was then dumped and the plates were washed twice with PBST and once with just PBS. After the extensive washing, 50ul of the substrate TMB (Sigma) was added to each well. The plates were set aside for about 10 min. while color development was allowed to take place. Once the positive control wells were adequately intense enough in color, compared to previous experience with colorimetric assays, determined visually, 50ul of 1 M sulfuric acid was added to each well to stop the color development. The plate’s absorbance was then read at 450nm by a Bio-Tek EL800X microplate reader (Bio-Tek Instruments, Winooski, VT).

**Cytokine Assay**

The supernatants from the cell adhesion assays performed earlier were assayed for the presence of certain cytokines and chemokines. Supernatants were allowed to thaw at room temperature. Manufactured Endogen ELISA kits were used to detect IL-8, IL-1α, IL-1β, and macrophage inflammatory protein (MIP-1α) all the kits were purchased from Pierce Chemical (Rockford, IL). Manufactured eBioscience kits were used to detect IL-6, and MCP-1. Manufactured DuoSet® ELISA kits from R & D Systems Inc. (Minneapolis, MN) were used to detect granulocyte macrophage-colony stimulating factor (GM-CSF) and eotaxin (CCL11). All of the assays were performed in accordance with the manufacture’s recommended protocols. Absorbance was read at 450nm by a Bio-Tek EL800X microplate reader.
Statistics

A total of twelve sets of cells each from a different passage (range from 4 to 12) but all from the same stock were used in all of the experiments. The first six sets of cells were all tested for adhesion molecule expression. Out of the six sets of cells, three sets were co-stimulated with house dust mite extract and TNF-α and three sets were only stimulated with house dust mite extract and not TNF-α. The six additional sets of cells were used to test for chemokine receptor expression. Out of those six sets of cells, three sets were stimulated with TNF-α and three were not stimulated with TNF-α.

The absorbance of each well was determined and its optical density was calculated. Each well of the 96 well plates used in these experiments had two more wells with the same concentration of extract and were tested for the expression of the same molecule. Each well of this group of three will be referred to as a replicate. The average of every group of replicates on each 96 well plate was then calculated. These averages were used to construct graphs that showed the pattern of expression or lack thereof for each individual molecule tested for compared to the other molecules also tested for with that set of cells. Another set of graphs compared the expression of the different adhesion molecules between TNF-α stimulated cells versus the non-TNF-α stimulated cells.

The standard deviation and standard error were calculated using the averages obtained from the three replicates. These calculations were done on each set of cells. The standard deviation of the results was used to calculate the standard error values. The standard error values were used to make error bars on the graphs. Standard error was used to calculate the length of the error bars because it showed the variability in the amount of absorbance between the three wells from that of the mean. The data obtained from the
different sets of cells that were used in this experiment could only be compared to each other in a qualitative way and not in a quantitative way due to the varied amount of cells used from each set.

The cytokine and chemokines data was processed in a very similar fashion to the adhesion molecule and chemokine receptor data, but only one set of graphs were produced. The graphs produced for the cytokine and chemokine data compared the expression of the cytokines and chemokines between TNF-α stimulated endothelial cells and non-TNF-α stimulated endothelial cells.
Results

Cell Adhesion Molecules

The HMVEC-d were assayed for ICAM-1, VCAM-1, and E-selectin by the ELISA procedure. The HMVEC-d were stimulated with three different concentrations of one of the two common house dust mite species *Dermatophagoides farinae* and *D. pteronyssinus*. After incubating for 12 and 24 hours the cells were assayed for the expression of adhesion molecules. In the second half of the experiment the HMVEC-d were stimulated with the house dust mite extracts as well as TNF-α and then assayed for the same adhesion molecules.

The primary antibody for all of the ELISAs that were run during the course of this research were titrated. From the titration it was found that a dilution of 1:500 produced the best absorbance numbers with this cell type. For the chemokine receptors a 1:100 dilution was used. However, even though the primary antibody was titrated the results for the expression of VCAM-1 and E-selectin had absorbance numbers below the limit of detection (~0.05 Abs) of the assay, putting the validity of the results in question.

ICAM-1 was constitutively expressed by HMVEC-d but both VCAM-1 and E-selectin were not expressed constitutively. TNF-α stimulation was able to significantly up regulate the expression of all three adhesion molecules. The TNF-α stimulation increased expression of the adhesion molecules by more than half. VCAM-1 and E-selectin expression was increased by 75% over the media controls (fig. 1).

The increasing dilutions of the house dust mite extracts caused a significant dose dependent increase in ICAM-1 expression by the HMVEC-d. This increased expression
was found to be slightly greater during the 24 hour incubation period then it was during the 12 hour incubation period. The *D. farinae* extract was able to stimulate more expression of ICAM-1 then the *D. pteronyssinus* extract was able to (fig. 3).

The house dust mite extracts had no noticeable effect on ICAM-1 expression by HMVEC-d when the cells began in an immunologically active state due to the addition of TNF-α. Co-stimulation with both house dust mite extract and TNF-α also canceled out the increased stimulation effect that *D. farinae* had over *D. pteronyssinus* that was seen in earlier experiments (fig. 4).
**Figure 1:** Expression of adhesion molecules by dermal endothelial cells after exposure to just tissue culture media (EBM-2) or tissue culture media and TNF-α for a 24 hour period. The averages of the EBM-2 data represent an n of 4. The averages of the EBM-2/TNF-α data represent an n of 2. All of the above points are significantly different at P≤0.01. Error bars represent ±SE.

**Figure 2:** Expression of chemokine receptors by dermal endothelial cells after exposure to just tissue culture media (EBM-2) or tissue culture media and TNF-α for a 12 hour and a 24 hour period. The averages of all the data represent an n of 6. All data points on the above graphs that have an asterisks are significant increases (P<0.05) from the media EBM-2 in the same incubation period. Error bars represent ±SE.
Figure 3: Expression of the cell adhesion molecule ICAM-1 from dermal endothelial cells upon exposure to house dust mite extract. Graphs a, b, and c represent three different sets of cells termed run 1, 2, and 3 respectively. All data points on the above graphs that have an asterisks are significant increases (P<0.05) from the media controls. Error bars represent ±SE.
Figure 4: Expression of the cell adhesion molecule ICAM-1 from dermal endothelial cells upon exposure to house dust mite extract as well as TNF-alpha. Graphs a, b, and c represent three different sets of cells termed run 1, 2, and 3 respectively. Media control for the 1st run received TNF-alpha in only 1 of the 3 wells during the 24 hour incubation period. All data points on the above graphs that have an asterisks are significant increases (P<0.05) from the media controls. # denotes data point based on calculations of 1 well. Error bars represent ±SE.
Since EBM-2 alone did not show any up regulation of VCAM-1 expression (fig. 1) by the cells it would be safe to assume that VCAM-1 is not constitutively expressed like ICAM-1. The media control wells of the non TNF-α stimulation experiments (fig. 5, and especially figure 5c) show almost no or very little VCAM-1 expression which provides further support for this observation.

The VCAM-1 data overall were not as clear as the ICAM-1 data. There was a small dose dependent increase in VCAM-1 expression upon exposure to house dust mites and tissue culture media alone (fig. 5a and c) however the absorbance numbers of the individual runs were low enough to put the accuracy of the results in question. Therefore no conclusions can be made on the expression pattern of VCAM-1 by HMVEC-d that were stimulated by house dust mite extract alone.

TNF-α is able to up regulate VCAM-1 expression by HMVEC-d (fig. 1), but when you add house dust mite extract to dermal endothelial cells that have already been stimulated with TNF-α (fig. 6), the house dust mite extract does not seem to have a large effect on VCAM-1 expressions. The absorbance numbers of the second and third runs (fig. 6b and c) of the TNF-α stimulated experiments were very low similar to the non-TNF-α stimulated experiments (fig. 5). The absorbance numbers of the first run (fig. 6a) were so much higher than the other two runs that the accuracy of any results received from the TNF-α stimulated experiments (fig. 6) should be questioned.

The low absorbance numbers observed in the VCAM-1 expression experiments (fig. 5 and 6) were not observed in the ICAM-1 expression experiments (fig. 3 and 4) indicating that the assay was functioning properly. The low absorbance numbers may
indicate that the HMVEC-d express very small amounts of VCAM-1 when stimulated by house dust mite extract or house dust mite extract and TNF-α.
Figure 5: Expression of the cell adhesion molecule VCAM-1 from dermal endothelial cells upon exposure to house dust mite extract. Graphs a, b, and c represent three different sets of cells termed run 1, 2, and 3 respectively. All data points on the above graphs that have an asterisks are significant increases (P<0.05) from the media controls. Error bars represent ±SE. Dotted line represents the limit of detection.
Figure 6: Expression of the cell adhesion molecule VCAM-1 from dermal endothelial cells upon exposure to house dust mite extract as well as TNF-alpha. Graphs a, b, and c represent three different sets of cells termed run 1, 2, and 3 respectively. Media control for the 1st run received TNF-alpha in only 1 of the 3 wells during the 24 hour incubation period. All data points on the above graphs that have an asterisks are significant increases (P<0.05) from the media controls. # denotes data point based on calculation of only 1 well. Error bars represent ±SE. Dotted line represents the limit of detection.
TNF-α stimulation of the endothelial cells is able to only cause a very slight increase in the expression of E-selectin (fig. 1). When the HMVEC-d were exposed to EBM-2 alone they showed no expression of E-selectin implying that E-selectin is not expressed constitutively (fig. 1). This observation is backed up by the fact that the media control wells of the non TNF-α stimulation experiments (fig. 7) showed very little to almost no expression of E-selectin.

The results of the E-selectin TNF-α stimulated experiments (fig. 7) and the non TNF-α stimulated experiments (fig. 8) had very low absorbance numbers similar to the results received in the VCAM-1 expression experiments (fig. 5 and 6). With absorbance numbers that match that of background; any conclusions made about expression patterns from the data may not be accurate.

Since the results of the VCAM-1 expression experiments (fig. 5 and 6) and the results of the E-selectin experiments (fig. 7 and 8) both showed very low absorbance numbers and the fact that the assay was demonstrated to be functioning properly indicate that HMVEC-d do express small amounts of E-selectin when stimulated by house dust mite extract or house dust mite extract and TNF-α.
Figure 7: Expression of the cell adhesion molecule E-selectin from dermal endothelial cells upon exposure to house dust mite extract. Graphs a, b, and c represent three different sets of cells termed run 1, 2, and 3 respectively. All data points on the above graphs that have an asterisk are significant increases (P<0.05) from the media control. # denotes data point based on calculations of only 2 wells. Error bars represent ±SE. Dotted line represents the limit of detection.
Figure 8: Expression of the cell adhesion molecule E-selectin from dermal endothelial cells upon exposure to house dust mite extract as well as TNF-alpha. Graphs a, b, and c represent three different sets of cells termed run 1, 2, and 3 respectively. Media control for the 1st run received TNF-alpha in only 1 of the 3 wells during the 24 hour incubation period. All data points on the above graphs that have an asterisks are significant increases (P<0.05) from the media control. # denotes data point based on calculation of only 1 well. Error bars represent ±SE. Dotted line represents the limit of detection.
Chemokine Receptors

The ELISAs for the chemokine receptors (CXCR-1, CXCR-2, and CCR-5) were run in much the same way as the ELISAs for the cell adhesion molecules. The biggest difference was obviously in the primary antibody that was used. The other difference in the two ELISAs was due to the fact that the primary antibodies for the chemokine receptors were not biotinylated like the primary cell adhesion antibodies were. This meant that a different type of secondary antibody was needed to properly bind with the primary antibody of the chemokine receptor ELISAs.

Both of the non TNF-α stimulated and the TNF-α stimulated plates of the third chemokine receptor run were ran at the same time. The non TNF-α stimulated plate and the TNF-α stimulated plate were ran at different times during the first and second runs. The reason that this fact is important is because several different observations can be made from the results of the third run of ELISAs that can not be made from the results of the first and second run of ELISAs.

TNF-α is able to stimulate increased expression of the chemokine receptors CXCR-1, CXCR-2, and CCR-5 by HMVEC-d slightly in both the 12 hour and the 24 hour incubation periods (fig. 2). CXCR-1 showed a higher amount of expression than the other receptors did in just EBM-2 as well as EBM-2/TNF-α, CCR-5 came in second and CXCR-2 was the lowest in the amount of expression but by a very close margin (fig. 2). This pattern in the amount of expression by CXCR-1, CXCR-2, and CCR-5 can also be qualitatively seen when you compare the third runs of both the non TNF-α and the TNF-α stimulation CXCR-1 experiments (fig. 9c and 10c) with the third runs of the non TNF-α and the TNF-α stimulation experiments of CXCR-2 and CCR-5 (fig. 11c, 12c, 13c, and
The scale for the former (fig. 9c and 10c) is an average of 1.5 absorbance units where as the average absorbance units for the latter (fig. 11c through 14c) is 0.5. This difference in scales of the chemokine receptors may also just be variability in the data and not a true effect of the expression of the receptors.

All of the chemokine receptors that were tested for in this research are constitutively expressed. Since the media control wells of all the non TNF-α stimulation experiments for CXCR-1, CXCR-2, and CCR-5 showed expression of those chemokine receptors (fig. 2, 9, 11, and 13) and since constitutive expression is indicated when a protein shows expression when there is no stimulation for it the deduction was made.

House dust mite extract does not have much effect on the expression of CXCR-1 by HMVEC-d (fig. 9). The second and third runs of the non TNF-α stimulation experiment (fig. 9b and c) showed a very slight but not very significant dose dependent increase in its expression. When the HMVEC-d were stimulated with TNF-α (fig 10) the expression of CXCR-1 seemed to show almost no difference from the HMVEC-d that were not stimulated with TNF-α (fig. 9).

All in all CXCR-2 expression showed pretty much the same pattern that expression of CXCR-1 did (fig. 11), that pattern being house dust mite extract not having any effect on CXCR-2 expression. The first run of the non TNF-α stimulation experiments (fig. 11a) showed a significant increase at the 25 ug/ml (D. farinae 24 hour data set) concentration as compared to the media control wells. The D. farinae 24 hour data set from that same run then showed a significant decrease in expression at the 200 ug/ml concentration over the 25 ug/ml concentration. Since this pattern of expression was only seen in that run and none of the other runs it can most likely be discounted.
House dust mite extract and TNF-α had very little effect on CCR-5 expression by HMVEC-d (fig. 13 and 14). There was very little significant increase in the amount of CCR-5 expression over the media controls in any of the runs.

There was also no discernable difference in the effects of *D. farinae* versus *D. pteronyssinus* on the expression of any of the chemokine receptors that were tested for.
Figure 9: Expression of the chemokine receptor molecule CXCR-1 from dermal endothelial cells upon exposure to house dust mite extract. Graphs a, b, and c represent three different sets of cells termed run 1, 2, and 3 respectively. All data points on the above graphs that have an asterisks are significant increases (P<0.05) from the media control. Error bars represent ±SE.
Figure 10: Expression of the chemokine receptor molecule CXCR-1 from dermal endothelial cells upon exposure to house dust mite extract as well as TNF-alpha. Graphs a, b, and c represent three different sets of cells termed run 1, 2, and 3 respectively. All data points on the above graphs that have an asterisk are significant increases (\(P<0.05\)) from the media control. Error bars represent ±SE.
Figure 11: Expression of the chemokine receptor molecule CXCR-2 from dermal endothelial cells upon exposure to house dust mite extract. Graphs a, b, and c represent three different sets of cells termed run 1, 2, and 3 respectively. All data points on the above graphs are significant increases (P<0.05) from the media control except for the data points that have an asterisks above them. Error bars represent ±SE.
Figure 12: Expression of the chemokine receptor molecule CXCR-2 from dermal endothelial cells upon exposure to house dust mite extract as well as TNF-alpha. Graphs a, b, and c represent three different sets of cells termed run 1, 2, and 3 respectively. All data points on the above graphs that have an asterisk are significant increases (P<0.05) from the media control. Error bars represent ±SE.
Figure 13: Expression of the chemokine receptor molecule CCR-5 from dermal endothelial cells upon exposure to house dust mite extract. Graphs a, b, and c represent three different sets of cells termed run 1, 2, and 3 respectively. All data points on the above graphs that have an asterisks are significant increases (P<0.05) from the media control. # denotes data point not based on calculations of all 3 wells. Error bars represent ±SE.
Figure 14: Expression of the chemokine receptor molecule CCR-5 from dermal endothelial cells upon exposure to house dust mite extract as well as TNF-alpha. Graphs a, b, and c represent three different sets of cells termed run 1, 2, and 3 respectively. All data points on the above graphs that have an asterisk are significant increases (P<0.05) from the media control. Error bars represent ±SE.
Cytokines

The ELISAs for the cytokines IL-6, IL-8, MCP-1, and GM-CSF were run differently than the adhesion molecule ELISAs and the chemokine receptor ELISAs were. When the cell adhesion ELISAs were run the supernatants were collected. Those collected supernatants were the testing solutions that the cytokine ELISAs were run on. Since the cytokines that were tested for in this research were all soluble molecules unlike the adhesion molecules and the chemokine receptors the plates had to be coated with a capture antibody to hold the cytokine in place so it could be detected.

It is clear that house dust mite extract significantly up regulates IL-6 expression by HMVEC-d in a dose dependent manner when there is no TNF-α stimulation (fig. 15a-c). When the cells have been stimulated with TNF-α (fig. 15d) there seemed to be a slight down regulation of IL-6. This down regulation can be seen in the second run of the TNF-α experiments (fig. 15e) as well, which shows a pretty drastic decline in IL-6 expression from the 100 ug/ml concentration of house dust mite extract/TNF-α to the 200 ug/ml concentration of house dust mite extract/TNF-α.

On the other hand there was a pretty large and significant dose dependent increase in IL-6 expression by the HMVEC-d in the third run of the TNF-α stimulated cells (fig. 15f). The first two runs of the TNF-α stimulated experiments (fig. 15d and e) were not conclusive enough to state that house dust mite extract/TNF-α is able to down regulate IL-6 production so it can be assumed that run 1 and 2 (fig. 15d and e) were flukes maybe caused by experimental error.

The expression of IL-6 matches that of ICAM-1 (fig. 3) and VCAM-1 (fig. 5) in the respect that *D. farinae* is able to stimulate IL-6 expression from non TNF-α
stimulated HMVEC-d to a much greater extent than *D. pteronyssinus* could (fig. 15a-c). When the HMVEC-d were stimulated with TNF-α the relationship in IL-6 expression by the two species is much less pronounced (fig. 15d-f). The third run of the TNF-α stimulation experiments (fig. 15f) did demonstrate some ability for *D. farinae* to cause a higher amount of expression of IL-6 than *D. pteronyssinus* could but that one experiment is not conclusive enough to alter the original observation. The second run of the TNF-α stimulation experiment (fig. 15e) was peculiar in the fact that there was almost no expression of IL-6 by the cells exposed to *D. farinae*. This lack of expression could be due to human error in the experimental procedure or maybe the original cell culture had a low cell number count.

There seems to be no discernable pattern in the effect of incubation time on the amount of IL-6 expression (fig. 15). Some of the runs of the IL-6 experiment (fig. 15a, b, and d) show that the dermal endothelial cells exposed to house dust mite extract for 24 hours expressed more IL-6 than the cells exposed to house dust mite extract for only 12 hours. However, when you look at the other runs (fig. 15c and f) they show that there was more IL-6 expression during the 12 hour incubation period. It may be that the 12 hour difference between a 12 hour incubation period and 24 hour incubation period is not enough time to show a difference in IL-6 expression.
Figure 15: The supernatants of the cell adhesion assays were tested for the presence of IL-6. Each set of graphs (i.e. a, d etc.) represent runs 1, 2, and 3 respectively with EBM-2 alone (a-c) versus EBM-2 and TNF-α (d-f). All data points on the above graphs that have an asterisks are significant increases (P<0.05) from the media control. Error bars represent ±SE.
Unlike IL-6, IL-8 is constitutively expressed by dermal endothelial cells (fig. 16a-c). This can be seen in the fact that the media control wells of run 1 and 3 of the non TNF-α stimulation experiments (fig. 16a and c) had a large amount of IL-8 expression. The media control wells of the second run (fig. 16b) did not show much expression this could be do to problems with the original supernatants from the adhesion molecule assays or for other unknown reasons. This lack of expression was seen in MCP-1 expression (fig. 17b) as well for similar unknown reasons.

The dose dependent increase in the expression of IL-8 by the HMVEC-d after house dust mite extract exposure is not as obvious as the dose dependent increase of IL-6 expression under the same conditions (fig. 16a and c). It seems that the HMVEC-d produce a large amount of IL-8 on their own with out the need for any type of stimulation (fig. 16a and c) evident by the fact that the amount of pg/ml of IL-8 produced in all three runs with or without TNF-α stimulation is much higher than that of IL-6. The second run (fig. 16b) did not show an obvious dose dependent increase in the expression of IL-8 after the HMVEC-d were exposed to *D. farinae* on the other hand when the cells were exposed to *D. pteronyssinus* the dose dependent increase pattern of expression was much more obvious.

It does not seem that TNF-α has a very large effect on IL-8 expression. The dose dependent increase in expression of IL-8 by the HMVEC-d seen with just house dust mite extract exposure (fig. 16a-c) was almost completely absent when the cells were incubated with TNF-α as well as the extract (fig. 16d-f). The second run of the TNF-α stimulation experiments (fig. 16e) showed a slight increase in the expression of IL-8 by the endothelial cells but that was not seen in the other two runs so it may be a fluke.
There is a very small difference in the amount of IL-8 expression by dermal endothelial cells that were exposed to *D. farinae* versus the amount of expression of IL-8 by dermal endothelial cells that were exposed to *D. pteronyssinus* (fig. 16). This difference in the expression pattern of IL-8 caused by the two different species of house dust mite is best demonstrated in the second run of the non TNF-α stimulation experiments (fig. 16b) the *D. farinae* extract caused an increase in the expression of IL-8 at the 25ug/ml concentration and that expression level stayed high at the 100ug/ml and 200ug/ml concentrations. The *D. pteronyssinus* extract caused a more casual increase in the expression level of IL-8 by the HMVEC-d. Looking at the IL-6 data (fig. 15) the difference in the effects the two species of house dust mites have on HMVEC-d was a lot more obvious than it was with the IL-8 expression data (fig. 16).

There is some variation in the expression of IL-8 from the 12 hour incubation period to the 24 hour incubation period (fig. 16). The first two runs of the IL-8 experiments (fig. 16a, b, d, and e) show that there was a smaller amount of IL-8 present in the 12 hour wells than there was in the 24 hour wells. This pattern can not be seen as well in the third run of the IL-8 experiments (fig. 16c and f), due to the fact that all of the data points in their respective concentrations are almost equal length. This could also be due to the fact that the amount of IL-8 expression may have been so high that it was beyond the detection level of the ELISA.
Figure 16: The supernatants of the cell adhesion assays were tested for the presence of IL-8. Each set of graphs (i.e. a, d etc.) represent runs 1, 2, and 3 respectively with EBM-2 alone (a-c) versus EBM-2 and TNF-α (d-f). All data points on the above graphs that have an asterisk are significant increases ($P<0.05$) from the media control. # denotes data points based on calculations of only 2 wells. Error bars represent ±SE.
MCP-1, like IL-8, showed constitutive expression in the non TNF-α stimulation experiments (fig. 17a-c), demonstrated by the fact that the media control wells of the non TNF-α stimulation experiments showed substantial expression of MCP-1 similar to the expression levels seen with IL-8 in the media control wells of IL-8’s non TNF-α stimulation experiments (fig. 16a-c). The second run of the non TNF-α stimulation ELISAs (fig. 17b) showed very little MCP-1 expression in the media control wells compared to the media control wells of the first and third runs (fig. 17a and c). This lack of expression was also seen in the second run of the IL-8 experiment (fig. 16b). This could mean that there was a problem with the supernatants of the original adhesion molecule ELISA or some other unknown reason.

In general MCP-1 expression by HMVEC-d increased significantly in a dose dependent manner upon exposure to house dust mite extract (fig. 17). The first two runs of the non TNF-α stimulated experiments (fig. 17a and b) showed a small leveling off of MCP-1 expression at the 200ug/ml concentration. This leveling off is not present in the third run (fig. 17c) probably because the highest concentration in that particular experiment was 150ug/ml. This may mean that a concentration of 200ug/ml of house dust mite extract maybe the HMVEC-d threshold of reactivity for MCP-1 expression. In order to confirm 200ug/ml of house dust mite extract as the threshold of reactivity an another assay would have to be run with house dust mite extract concentrations higher than 200ug/ml used.

The dose dependent increase in MCP-1 expression is not as clear in the TNF-α stimulation data (fig. 17d-f). The experiments just seemed to be more varied in their results. The first and third runs of the TNF-α stimulation experiments showed a slight
increase in expression of MCP-1 (fig. 17d and e). There is a dip in expression at the 25ug/ml concentration in the third run of the TNF-α stimulation experiment (fig. 17e). This dip has been observed in the IL-6 (fig. 15e), IL-8 (fig. 16e), and GM-CSF experiments (fig. 18e) indicating that there may have been a low cell number count in the wells of the original adhesion molecule assay or a simple error in the collecting of the supernatants.

The differences between the effects that *D. farinae* and *D. pteronyssinus* have on the expression of MCP-1 by the HMVEC-d are more obvious than they were with the IL-8 expression data (fig. 16). The *D. farinae* extract is clearly able to stimulate more expression of MCP-1 than the *D. pteronyssinus* extract. The second run of the MCP-1 non TNF-α stimulation experiments (fig. 17b) showed a similar expression pattern to the second run of the IL-8 non TNF-α stimulation experiments (fig. 16b). The second run showed that the expression of MCP-1 by the HMVEC-d after exposure to *D. farinae* started out high and remained high at all three concentrations of house dust mite extract used. The expression level of MCP-1 by the HMVEC-d after exposure to *D. pteronyssinus* started low but increased with increasing concentrations of house dust mite extract.

On the other hand, both of the non TNF-α stimulated data and the TNF-α stimulated data show that there was increased expression of MCP-1 during the 24 hour incubation period than there was during the 12 hour incubation period. This pattern was also seen in IL-8 expression (fig. 16). This increase in expression during the 24 hour incubation period is much more pronounced with MCP-1 expression than it is with IL-8 expression.
Figure 17: The supernatants of the cell adhesion assays were tested for the presence of MCP-1. Each set of graphs (i.e. a, d etc.) represent runs 1, 2, and 3 respectively with EBM-2 alone (a-c) versus EBM-2 and TNF-α (d-f). All data points on the above graphs that have an asterisk are significant increases (P<0.05) from the media control. # denotes data point based on calculations of only 2 wells. Error bars represent ±SE.
GM-CSF is not constitutively expressed (fig. 18a-c). There is hardly any expression of GM-CSF in the media control wells of any of the runs in the non TNF-α stimulation experiments for GM-CSF (fig. 18a-c). On the other hand the control wells of all of the runs for the TNF-α stimulation experiments (fig. 18d-e) showed some expression of GM-CSF by the HMVEC-d. Since the only stimulation that those cells have received is TNF-α we can deduce that TNF-α is able too stimulate GM-CSF expression on its own.

House dust mite extract was able to significantly increase expression of GM-CSF by HMVEC-d in a dose dependent manner (fig. 18a-c). This dose dependent increase in GM-CSF expression is best demonstrated in the third run of the non TNF-α stimulation experiments (fig. 18c). The first two GM-CSF runs were not conclusive because the supernatants from the original adhesion molecule ELISA were running low and thus the amount tested was less than a full dose.

The TNF-α stimulated cells (fig. 18d-f) show a significant dose dependent increase of GM-CSF expression just like the non TNF-α stimulated cells (fig. 18a-c) did. The biggest difference between the TNF-α stimulated cells (fig. 18d-f) and the non TNF-α stimulated cells (fig. 18a-c) is the pattern of GM-CSF expression of *D. farinae* versus *D. pteronyssinus*. In the non TNF-α stimulated cells (fig. 18a-c) the *D. farinae* seem to cause a much larger amount of expression of GM-CSF than *D. pteronyssinus* did. With the TNF-α stimulated cells (fig. 18d-f) the difference in expression by the two species is almost completely absent. In fact the second run of the TNF-α stimulation experiments (fig. 18e) showed that *D. pteronyssinus* caused a higher amount of expression of GM-CSF than *D. farinae* did.
Figure 18: The supernatants of the cell adhesion assays were tested for the presence of GM-CSF. Each set of graphs (i.e. a, d etc.) represent runs 1, 2, and 3 respectively with EBM-2 alone (a-c) versus EBM-2 and TNF-α (d-f). All data points on the above graphs that have an asterisks are significant increases (P<0.05) from the media control. # denotes data points not based on calculations of all 3 wells. Error bars represent ±SE.
## Result Summary

### Adhesion Molecules

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<th>Parameter tested</th>
<th># of Replicates</th>
<th>Constitutive expression</th>
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<th>TNFα alone</th>
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### Chemokines and Cytokines

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### Chemokine receptors

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*Showed a dose dependent relationship.  
+Increase was slight but significant.
Discussion

Atopic dermatitis (AD) is a chronic inflammatory skin disease that often plagues its patients with painful eczematous skin eruptions (Finlay et al. 1980). People worldwide suffer from AD, but it is more common in children, with a 10% to 20% prevalence compared to adults where it only has a 1% to 3% prevalence (Larsen and Hanifin 2002). AD is often triggered in people after exposure to a number of environmental factors one of them being house dust mite allergens (Tupker et al. 1996). Since dermal endothelial cells have an important role in the regulation of inflammation they in turn are very important in the pathogenesis of AD (Steinhoff et al. 2006). The response of dermal endothelial cells to exposure of house dust mites has not been well studied. In this research I attempted to achieve a better understanding of the endothelial cell’s response to house dust mites by exposing human dermal microvascular endothelial cells (HMVEC-d) to house dust mite extract and evaluating the effects the extract had on the expression pattern of certain proinflammatory cytokines, adhesion molecules, and chemokine receptors.

Dermal endothelial cell’s ability to express adhesion molecules, certain cytokines, as well as chemokines and their receptors give the dermal endothelial cells the ability to control the migration of leukocytes into the dermis (Hillyer et al. 2003). This control over leukocyte migration allows the inflammatory process to progress in an orderly and timely fashion. For instance, inflammatory cells such as macrophages and neutrophils are brought into the dermis first. Later T and B cells of the adaptive immune system migrate into the dermis (Osborn 1990). When allergenic molecules initiate an allergic dermal
immune response eosinophils and mast cells are brought into the site of inflammation as well.

This controlled infiltration of leukocytes from the blood vessels into the dermis is performed in three phases: rolling, firm adhesion/activation, and diapedesis. During the rolling phase, the leukocytes roll along the endothelial wall of the post capillary venule. This rolling movement occurs because of the formation of low affinity bonds between selectin ligands on the leukocytes surface and E-selectin on the endothelial cells surface. The continual breaking and reforming of these low affinity bonds between the two cells results in the leukocytes rolling across the endothelium (Osborn 1990, Springer 1994).

Once E-selectin expression and leukocyte rolling is initiated, the endothelial cells begin to express the Ig domain adhesion molecules ICAM-1, ICAM-2, VCAM-1, and VCAM-2. Expression of these molecules results in firm adhesion between the leukocytes and the endothelial wall. This firm adhesion is due to the formation of high affinity bonds between the integrins on the plasma membrane of the leukocytes and the Ig domain adhesion molecules on the plasma membrane of the endothelial cells (Granger and Kubes 1994).

Once the adhesion molecules have been expressed and the leukocyte is firmly adhered to the endothelium, the endothelial cell becomes activated. Once activated, the expression of chemokine receptors on the surface of the endothelial cells and the emigrated leukocytes is increased. The activation of endothelial cells also leads to their production of the chemokines IL-8 and MCP-1 (Lukacs et al. 1995, Kinoshita et al. 2005). Once the leukocyte is firmly adhered to the activated endothelium through the
binding of adhesion molecules and their ligands on the surface of the incoming leukocyte, the leukocyte can then be passed between the cells and into the dermis.

The results from this study showed that house dust mite extract caused an extensive up regulation in the expression levels of several proinflammatory molecules and adhesion molecules that are key players in extravasation of inflammatory and immune cells. The adhesion molecule ICAM-1 showed a significant dose dependent increase in its level of expression by HMVEC-d after the cells were stimulated with house dust mite extract as well as TNF-α compared to the constitutive level of expression that ICAM-1 displayed before the HMVEC-d were stimulated with the extract or with TNF-α. The other adhesion molecules that were tested for in the course of this research, VCAM-1 and E-selectin, showed very little expression by HMVEC-d after house dust mite extract and TNF-α stimulation. The expression levels of IL-6, IL-8, MCP-1, and GM-CSF by HMVEC-d also showed a significant dose dependent increase after exposure to the extract. The chemokine receptors CXCR-1, CXCR-2, and CCR-5 were constitutively expressed by HMVEC-d and the house dust mite extracts and TNF-α demonstrated very little ability to alter that expression level. HMVEC-d showed no constitutive expression of the proinflammatory molecules IL-1α, IL-1β, and eotaxin and stimulation with house dust mite extract did not induce secretion of these cytokines. Stimulation with TNF-α also did not induce secretion of these proinflammatory cytokines. Taken together, these data suggest that HMVEC-d are unable to express those proinflammatory cytokines even with inflammation causing substances present or human error (i.e. faulty measurements or improper incubations periods) lead to a lack of expression by any of these cytokines.

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In order to see the expression of these adhesion molecules and proinflammatory cytokines by the endothelial cells, the length of the incubation periods had to be chosen carefully. If the incubation was too long than all of the adhesion molecules and cytokines that I wanted to observe would have been expressed and than degraded before I had a chance to see them. If the incubation period was too short than the molecules that I wanted to observe would not have been expressed yet. Twelve hour and 24 hour incubation periods were chosen due to previous research that used a 12 hour and 24 hour incubation period in a very similar context that I was going to use (Elder et al. 2006). In that study they detected expression by E-selectin in both incubation periods and the same with ICAM-1 and VCAM-1. The timing in the expression pattern of the adhesion molecules and cytokines is important to understanding the effects that house dust mite extract has on dermal endothelial cells.

E-selectin and P-selectin are the first major adhesion molecules to be expressed by the endothelial cell once it is activated. P-selectin was not focused on in this research because of the fact that its observable pattern of expression only lasts for about 30 to 40 minutes after the initial activation of the endothelial cell (Hattori et al. 1989). Expression of E-selection occurs after P-selectin expression but before the expression of the Ig-domain adhesion molecules ICAM-1, ICAM-2, VCAM-1, and VCAM-2. ICAM-1 does not reach its peak of expression until later in the lymphocyte recruitment process at about the 24 hour mark (Leeuwenberg et al. 1992, Kluger et al. 1997) and VCAM-1 reaches its peak of expression sooner than ICAM-1, at the 6-12 hour mark (Kaplanski et al. 1998). VCAM-1 expression data from Kaplanski et al. 1998 differs from the results that were obtained in my own research. The results of this study showed that there was more
expression of E-selectin by HMVEC-d after the 12 hour incubation period than there was after the 24 hour incubation period. On the other hand expression of ICAM-1 and VCAM-1 by HMVEC-d was shown to be much greater after the 24 hour incubation than there was after the 12 hour incubation. This difference in kinetics of the adhesion molecules between Kaplanski’s study and my own research is that Kaplanski used human umbilical vein endothelial cells and thrombin as the stimulating agent where as I used dermal human microvascular endothelial cells and house dust mite extract as the stimulating agent. This observation of adhesion molecule expression timing further supports the idea that E-selectin is expressed earlier in the lymphocyte recruitment process than ICAM-1, ICAM-2, VCAM-1, or VCAM-2.

The relationship between house dust mite exposure to the skin and the subsequent up-regulation of proinflammatory cytokines and adhesion molecules by dermal endothelial cells may not be as directly related to each other in vivo as they have been observed in my own in vitro study. In vivo, the bio-active and allergic molecules from house dust mite extract are not exposed directly to the endothelium like they are in cultured cells. During in vivo experiments the allergenic or bio-active molecules that penetrate the skin will encounter the many resident cell types of the epidermis and dermis such as keratinocytes, Langerhan cells, and fibroblasts as these molecules migrate to the endothelium. After the resident skin cells are activated by exposure to the allergenic or bio-active molecules, the cells may release the proinflammatory molecules IL-1, TNF-α, IL-8, and IL-6 (Boxman et al. 1996). These proinflammatory molecules in turn activate the endothelium to begin lymphocyte attachment before the endothelial cells ever encounter the allergenic or bio-active molecules that originally started the process (Chu
The activation of the endothelium by TNF-α in these in vivo studies is contradictory to the results that were made during my own in vitro study which showed that TNF-α was not able to alter the expression level of IL-8 and IL-6 in any substantial way. The differences in the results from the two studies may be because Chu conducted his experiments in vivo so multiple proinflammatory cytokines and not just TNF-α were in play and could have activated the endothelial cells to produce IL-8 and IL-6.

In this study we observed increased expression of IL-8 by HMVEC-d upon exposure to house dust mite extract. This increase in expression is significant because the chemokine IL-8 and its receptors CXCR-1 and CXCR-2 are important in the inflammation process. IL-8 has been shown to be a neutrophil and T-cell chemoattractant and has also demonstrated an ability to increase vascular permeability by Rac (a G-coupled intracellular signaling protein) activation and subsequent gap formation (Schraufstatter et al. 2001). IL-8’s chemoattractant, permeability modifying, and Rac activating abilities are all crucial to the efficient eradication of the antigenic molecules in house dust mite extracts when they have found their way into the dermis. IL-8’s chemoattractant ability is important in the inflammatory process because it attracts inflammatory cells to the endothelium by setting up a chemical gradient. Once the neutrophils and the T-cells reach the endothelium, the endothelial cells retract from each other and increase gap formation which allows for easier movement for those cells entering the dermis.

Up-regulation of IL-8 by endothelial cells during inflammatory conditions has also been observed in other situations such as during infection by R. rickettsii (Clifton et al. 2005) and after endothelial activation by mast cell’s tryptase via PAR-2 receptor (Lu and Morris 2004).
et al. 2005). Some studies have shown an increase in IL-8 by endothelial cells after exposure to scabies mite extract as well (Elder et al. 2006). *In vitro* this chemokine is up-regulated by other resident skin cell types such as dendritic cells, keratinocytes, fibroblasts, and mast cells after the cells were exposed to inflammation causing substances (Moller et al. 1993, Arlian et al. 2004, Pastore et al. 2004). During the early phase of the inflammatory process, the IL-8 that the endothelial cells uses to recruit leukocytes to the correct area of the endothelium is not produced by the endothelial cells themselves. The initial source of IL-8 must be from the other resident skin cell types such as keratinocytes and fibroblasts (Moller et al. 1993, Pastore et al. 2004).

The results from this study showed that unlike the chemokine receptor’s ligands IL-8 and MCP-1, the chemokine receptors CXCR-1, CXCR-2, and CCR-5 did not show any change in their expression level by HMVEC-d after exposure to house dust mite extract. The chemokine receptors CXCR-1 and CXCR-2 are used by the endothelial cell to bind IL-8 and create a chemoattractant gradient that brings leukocytes to the endothelium (Carlos and Harlan 1994). CXCR-1 and CXCR-2 are also expressed on the surface of leukocytes. Once IL-8 binds to the receptors on the leukocyte’s surface the cell goes through changes such as actin polymerization (Norgauer et al. 1994), to allow it to migrate through the endothelium, as well as the oxidative burst, to combat the inflammatory causing agents once it is in the dermis (Schraufstatter et al. 2001). Therefore, house dust mite exposure to the skin of a sensitized person can result in a strong inflammatory response because of products from these mites can induce increased expression of IL-8 and IL-6.
Another important proinflammatory cytokine that is up regulated by HMVEC-d after exposure to house dust mite extract is IL-6. There are many sources of expression of the cytokine IL-6 in the dermis and epidermis during the inflammatory process. Some studies where scabies mites were used as the stimulating agent showed an increase in IL-6 expression by keratinocytes, fibroblasts, and peripheral blood mononuclear cells (PBMC) (Arlian et al. 2003, Arlian et al. 2004). IL-6 has also shown an ability to act as an amplifying molecule for the inflammatory response by binding to PBMCs and signaling the production of even more IL-6 (Neuner et al. 1991). Most of the IL-6 produced in this way makes its way to the endothelium where it causes endothelial cells to increase their adhesiveness for leukocytes by up-regulating the expression of adhesion molecules on the endothelial cell’s surface (Watson et al. 1996). The results from this study showed that the endothelium itself is also a possible source of IL-6 since the presence of the house dust mite extracts lead to a similar increase in IL-6 expression by HMVEC-d cells that was seen in keratinocytes, fibroblasts, and PBMCs stimulated with scabies mite extracts.

This study also evaluated the effects that house dust mite extract had on the expression pattern of the proinflammatory cytokine granulocyte macrophage-colony stimulating factor (GM-CSF) by HMVEC-d. The data presented in this study showed that GM-CSF is up-regulated in a dose-dependent manner by dermal endothelial cells when they are stimulated by either house dust mite extract alone or house dust mite extract and TNF-α. GM-CSF acts as a growth factor for hematopoetic precursors, while also playing a role in the migration and proliferation of endothelial cells (Bussolino et al. 1989). GM-CSF’s role in migration and proliferation is especially important in angiogenesis (the
process of blood vessel formation) (Bussolino et al. 1991). Angiogenesis is essential in wound healing and in the pathophysiology of dermal diseases such as atopic dermatitis (Agha-Majzoub et al. 2005).

In other words, GM-CSF does not play a role in the early phase of the inflammatory process like IL-6. GM-CSF is expressed by a number of cell types such as fibroblasts and monocytes (Vellenga et al. 1988, Pang et al. 1994), but GM-CSF expression by those cells does not occur until the proinflammatory cytokines IL-1, IL-6, and TNF-α signal the cells to do so. Endothelial cells work in a similar way, they do not express GM-CSF until activated by proinflammatory cytokine or house dust mite extracts (Huleihel et al. 1993, Shannon et al. 1997). This delay in the expression of GM-CSF during inflammation makes sense if you consider GM-CSF role in the maturation of tissue macrophages and granulocytes as well as its role in wound healing. Maturation of hematopoietic precursors in to tissue macrophages and granulocytes and the process of wound healing cannot begin until after the initial activation of the resident dermal cells fibroblasts, keratinocytes, and endothelial cells by foreign antigenic/allergenic particles and/or proinflammatory cytokines. Therefore, even though house dust mites were shown to increase GM-CSF expression from endothelial cells in vitro, in vivo the increased expression of GM-SCF must not occur very quickly after the dermis initially encounters the antigenic particles of house dust mites.

It was interesting to note that even though the two species of house dust mites used in this study were from the same genus and therefore were genetically and morphologically very similar to each other, the two extracts were still able to cause differences in the expression pattern of a number of different molecules. The presence of
Dermatophagoides farinae extract was able to elicit expression of higher amounts of the adhesion molecules ICAM-1 and VCAM-1 and secretion of higher amounts of IL-6, MCP-1, and GM-CSF by HMVEC-d than the Dermatophagoides pteronyssinus extract.

At this time, it is not clear how or why the two species gave different results but this was a consist pattern observed throughout the experiments. It does not seem likely that the documented groups of allergens most commonly found in house dust mite extracts are the cause of the differences in the effects the two extracts have on HMVEC-d, due to the fact that most of the grouped allergens from the two species share such a high degree of sequence homology reviewed by (Thomas et al. 2002). The first major group of allergens from the two species (Der p 1 and Der f 1) share an 81% similarity in their sequence homology (Dilworth et al. 1991). The group two allergens (Der p 2 and Der f 2) share an even higher degree of sequence homology 88% (Thomas et al. 2002). The other less characterized components of house dust mite extract, such as the non-allergenic/antigenic bioactive molecules, might be the source of the differences in the reactions the two species of house dust mites have on HMVEC-d.

The data from the three different runs of the adhesion molecule ELISAs and the cytokine ELISAs showed a great deal of variation in their scales of absorbance. The first run usually showed the highest absorbance numbers, the second run usually showed the lowest absorbance numbers, and the third run was usually between there. There are several factors that could lead to this kind of variation in the data. The number of cells originally plated affects the amount of expression seen when the ELISA is run. The cell numbers started out very high for the first and second run of the non TNF-α stimulation experiments the third run started out with a much smaller cell count. The lack of a
standard time period for color development after the addition of the TMB substrate could also account for the variation in the data. The house dust mite extract used for the first two runs was different from the extract used in the last run. The difference in extracts used most likely did not affect the expression of the molecules differently due to the fact that both extracts were made the same way and even though they had different protein concentrations they were diluted down to the same protein concentration when they were added to the cells. The pattern of variation in the data cannot be explained by the occurrence of just one of these factors it must be a combination of different factors that lead to the observed variation in the data.

An important area of future research should be to determine the actual molecules in mite extract responsible for stimulating endothelial cells. Another would be to identify the receptors on the endothelial cells that respond to the extract. Both are areas of research that would help in understanding the role of house dust mites in A.D. Thus far, some of the work that has been done to find the point of contact between the molecules of the house dust mite extract and epithelial cells has pointed to protease activated receptors (PARs), no research has been done in this respect with endothelial cells but the PAR-2 receptor has been located on the surface of human dermal microvascular endothelial cells (Shpacovitch et al. 2002). It has been demonstrated that serine and cysteine protease allergens in house dust mite extract (groups 1, 3, and 9) are able to bind to and activate PARs (Sun et al. 2001, Asokananthan et al. 2002). PAR-2, one of four characterized PARs, has been shown to have proinflammatory effects during cutaneous inflammation in mice and humans \textit{in vivo} (Seeliger et al. 2003). Activation of PARs located on epithelial cells has been shown to lead to the expression of proinflammatory molecules
like GM-CSF, IL-6, IL-8, and MCP-1 (Sun et al. 2001, Asokananthan et al. 2002). In my own research, the cytokines GM-CSF, IL-6, IL-8, and MCP-1 were also shown to increase in expression by HMVEC-d after house dust mite extract exposure. The similarity between the expression pattern of certain cytokines after PAR activation and house dust mite extract stimulation suggests that it is the PARs on the surface of the HMVEC-d that are being activated by the molecules of house dust mites to produce the increase in expression of adhesion molecules and cytokines that was observed in my research. More research needs to be done to complete a working model for house dust mite extract activation of PARs located on the surface of HMVEC-d.

There is a large difference in the expression pattern of certain adhesion molecules and chemokines when you expose the related scabies mite extract to HMVEC-d versus exposure of house dust mite extract to the same cell type. Scabies mites, unlike their phylogenetic relative house dust mites, cause a decrease in the expression of E-selectin, VCAM-1, and IL-8 even after TNF-\(\alpha\) has stimulated those proinflammatory molecules to observed maximal levels of expression (Elder et al. 2006). This down regulation can partially explain the ability scabies mites have to delay the human immune system from responding for four to eight weeks after the initial infestation (Kemp et al. 2002). The expression level of E-selectin, VCAM-1, and IL-8 by HMVEC-d after house dust mite extract exposure is increased leading to an immediate allergic inflammatory reaction.

Even though scabies mites and house dust mites are phylogenetically closely related, scabies mites are obligate parasites where as house dust mites are facultative parasites. The difference in the parasitic relationships that the two species of mites have with humans can potentially explain the reason why there is a difference in the effects
that scabies mites and house dust mites have on HMVEC-d. The obligate parasite, scabies mite, cannot complete its life cycle without a host. On the other hand house dust mites being facultative parasites do not need a host to complete their life cycle. House dust mites are still considered parasites of human because of the fact that they need human skin scales to survive while providing only detrimental effects to humans in the form of allergies and allergic diseases. In order for scabies mites to survive long enough in the stratum corneum of human skin to complete its life cycle they had to evolve a method to delay the human immune system from killing it too early. House dust mites on the other hand had no need to evolve such mechanisms.

In conclusion, this study has demonstrated that molecules of house dust mite extracts are able to activate microvascular endothelial cells to produce increases in the expression of certain molecules that are involved in the development of inflammation. These mites induced HMVEC-d to secrete the cytokine IL-6 and the chemokines IL-8 and MCP-1 as well as increase the expression of the adhesion molecules ICAM-1, VCAM-1, and E-selectin.
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