AN ABSTRACT OF THE THESIS OF

Jennifer L. Sargent for the degree of Master of Science in Veterinary Science presented on June 2, 2015

Title: Ulcerative Dermatitis in C57BL/6 Mice

Abstract approved:

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Abstract

Ulcerative dermatitis (UD) is a common condition in C57BL/6 mice that is poorly understood and challenging to treat. Inconsistently there have been reports of an increased incidence of the disease in female mice, mice exposed to certain diets, mice of advanced age, and in several seasons. These inconsistencies indicated a need for a systematic review to better assess the evidence for commonly cited UD risk factors and treatments. The aims for the systematic review were to assess the quality of evidence for both: 1) commonly cited risk factors for UD, specifically sex, age, season, and diet; and 2) reported UD treatments. A search of three electronic databases was performed and articles were evaluated using previously published criteria for assessing methodological quality. Dietary factors, particularly caloric restriction, appear to have an effect on UD risk. Female sex was associated with an increased risk of UD in some studies, particularly diet studies, but not in others. Also, UD was seen most commonly in mice between 14 and 24 months of age in the studies reviewed. The role of season was not assessed in any of the articles that met the inclusion criteria. Of the three publications that evaluated UD treatments only one had an untreated or alternative therapy control. Further research is needed to explore epidemiologic aspects of UD and to compare treatment options.

While early investigations into UD focused on the possibility of a primary dermatopathology (e.g. vasculitis of cutaneous vessels or follicular dysplasia) several recent studies have advocated scratching behavior as a primary driver for UD. The aim of the second study was to assess whether B6 mice demonstrate excessive scratching behavior under resting conditions or when provoked by epidermal barrier disruption compared to DBA/2, BALB/c, and ICR mice. The behavior of the mice was videotaped in observation chambers and reviewed for scratching frequency and duration both prior to and following tape stripping to initiate epidermal barrier injury. In addition, a spray test was performed as this test was previously associated with future UD development in B6 mice. In contrast to the hypothesis, the B6 mice did not scratch significantly more frequently, they did not have more long duration scratching events, and they did not have a higher median scratching duration for the long scratching events. In fact, the B6 mice showed the least scratching frequency and duration under many conditions. B6 mice demonstrated a statistically significant increase in scratching behavior following epidermal barrier disruption but the increased scratching did not surpass the rate or duration of scratching seen in the other genotypes of mice tested. Although the experimental results failed to support the hypothesis, the low scratching frequency and duration of B6 mice has interesting implications for the role of scratching in UD development.

Ulcerative Dermatitis in C57BL/6 Mice

by
Jennifer L. Sargent

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented June 2, 2015
Commencement June 2015

Master of Science thesis of Jennifer L. Sargent presented on June 2, 2015.

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Jennifer L. Sargent, Author

ACKNOWLEDGEMENTS

I would like to thank my advisor and mentor Dr. Helen Diggs for her guidance and abundant support.

I would also like to extend my thanks to the Laboratory Animal Resources Center staff for the diligent care of my study animals. Also thanks for keeping the heckling to a minimum as I reviewed hours and hours of videos of mice in my darkened office.

I would like to acknowledge my committee members, Drs. Wendy Baltzer, Luiz Bermudez, Michelle Kutzler, and Christiane Löhr, for their time and insightful suggestions.

Thanks go to Dr. Kathy Magnusson for allowing me to use her laboratory's behavior suite for recording my study animals.

Finally, I would like to express my sincere appreciation to my family and friends for their incredible emotional support and understanding. I promise this will be my last degree! Probably…

CONTRIBUTION OF AUTHORS

Dr. Nathan Koewler assisted with the review of articles for content and manuscript editing for Chapter 2. In addition to her general mentoring and support, Dr. Helen Diggs was involved in data collection for Chapter 3.

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Chapter 1: General introduction

Overview

The most commonly used strain of mouse in biomedical research, the C57BL/6 (B6) mouse, is predisposed to a condition known as ulcerative dermatitis (UD). This condition is characterized by pruritic ulcerative skin lesions that are frequently progressive despite treatment and may result in debilitating scarring (Andrews et al. 1994, Duarte-Vogel and Lawson 2011, Kastenmayer et al. 2006, Lawson 2005). UD is considered quite common although reported prevalence varies widely in the literature. In one retrospective study that examined the prevalence of UD in specific animal rooms of their facility, 2% of male B6 mice developed UD while 6% of female B6 mice developed UD over the two year study period (Kastenmayer et al. 2006). However, the lifetime incidence was likely underestimated due to the removal of animals from that population for colony management or research related reasons prior to the typical age of disease onset. In contrast, a prospective cohort study of male and female B6 mice sacrificed at one of two study end-points found an UD prevalence of 6% in the 13 month cohort and 27% and 35% (males and females, respectively) in the 19 month cohort (Hampton et al. 2012). Others have reported UD prevalence as the percentage of health concerns reported to the veterinary staff at their institution. This number was also variable, ranging from 17-35% depending on the citation (Lawson et al. 2005, Marx et al. 2013, Williams-Fritze et al. 2011). By either measure UD is a substantial cause of morbidity in laboratory mice.

Ulcerative dermatitis can have devastating consequences for animal health and research. From an animal welfare perspective the severe pruritus and skin wounds that are refractory to treatment provide a significant source of distress for affected mice. Also, mice with this condition may be unsuitable for their intended research purpose due to confounding pathology

associated with UD. Due to these animal welfare and research concerns, many mice with UD are ultimately euthanized. The diagnosis and treatment of UD with marginal success, delays in research, and animal losses drain the resources of laboratory animal care departments and investigators. Certain types of research are more likely to be disrupted by spontaneous UD, particularly aging and life span studies, as UD is most common in mice over 1 year of age (Andrews et al. 1994, Kastenmayer et al. 2006, Mader et al. 2010).

Despite the impact of UD in laboratory animal medicine the condition remains poorly understood. The etiology and pathogenesis are largely unknown leaving veterinarians and animal care staff without reliable prevention measures or treatments. My frustration dealing with this common yet poorly understood condition in the clinical setting lead me to pursue UD research for my thesis project. The following work is intended to advance the current knowledge of UD in the hopes of taking an incremental step forward in understanding one of the most frustrating diseases encountered in laboratory animal medicine.

Literature review

<u>Ulcerative dermatitis</u>

Typical clinical history

UD is most commonly seen in mature mice (Andrews et al. 1994, Mader et al. 2010), although some mice may develop UD prior to 6 months of age (Kastenmayer et al. 2006). Affected strains include the C57BL/6, mutant strains on this background, and closely related inbred strains such as the C57BL/10 (Fox et al. 2002, Kastenmayer et al. 2006, Stowe et al. 1971). There are several well recognized substrains of B6 mice developed by breeding closed

colonies of B6 mice at different institutions resulting in genetic drift and minor genetic variations (described in Appendix A). It has been suggested that different substrains are more commonly affected than others, specifically that the C57BL/6J substrain may have a higher prevalence or earlier onset than the C57BL/6Crl, but this hypothesis has not been directly tested (Hampton et al. 2012, Sundberg et al. 2011). A central centrifugal cicatricial alopecia (CCCA)-like condition has been described in young C57BL/6J mice and may appear similar to UD (Sundberg et al. 2011). Confusion between the two conditions may account for some reports of substrain related differences regarding how UD progresses and appears clinically. Several reports suggest that there is an increased incidence in female mice (Kastenmayer et al. 2006, Sundberg et al. 1994). A seasonal component has also been reported with one institution reporting an increase in cases in the summer and another that found UD to be more common in the spring and autumn (Kastenmayer et al. 2006, Sundberg et al. 1994).

Gross lesion appearance and progression

Affected mice are typically reported to veterinary staff for skin ulcers associated with moderate to severe pruritus. The condition is considered to have a sudden onset, although small excoriations may be overlooked early in the course of the disease (Andrews et al. 1994, Hampton et al. 2012). Typical lesion distribution includes the head and dorsal cervicothoracic region (Hampton et al. 2012, Lawson et al. 2005). Lesions may occasionally be seen more caudally or even on the limbs. Necropsies of UD affected mice may reveal peripheral lymphadenopathy although this is not reliably appreciated on physical exam (Hampton et al. 2012, Kastenmayer et al. 2006). UD progression has concisely been described as follows (Andrews et al. 1994):

"The lesions began as single or multiple small crusts in the skin between the scapulae or on the dorsum of the neck. These lesions were intensely pruritic and rapidly worsened to become irregularly shaped ulcers. Most commonly, the lesions spread laterally and caudally. Occasionally, the lesions extended cranially to affect the base of the ears or cranioventrally to affect the ventral cervical skin."

Spontaneous resolution can occur but the recovery rate is low (Andrews et al. 1994, Ezell et al. 2012, Lawson et al. 2005, Williams-Fritze et al. 2011). Even in the event the ulcerations resolve severe wound scarring and contracture may occur, contributing to the overall poor prognosis of UD (Andrews et al. 1994, Lawson et al. 2005).

Diagnosis

No uniform diagnostic criteria for UD have been adopted. Diagnosis is typically achieved by a combination of lesion character in mice of an appropriate strain or background and exclusion of differential diagnoses (Hampton et al. 2012, Kastenmayer et al. 2006). As described above the lesions are most commonly ulcerations of varying size and depth which are most commonly located on the interscapular region, neck, and pinna in B6 mice and related strains and genetically engineered mice. Acariasis can result in skin ulcers and B6 mice are particularly susceptible to this manifestation of mite infestation (Dawson et al. 1986). Therefore, mite associated ulcerative dermatitis is an important differential to consider for the UD syndrome, although most facilities exclude these parasites through rigorous screening and treatment. Fight wounds may also appear similar to UD lesions and can be a reasonable differential diagnosis, especially in adult male mice. Fight wounds, which are most commonly seen as nonprogressive multifocal excoriations around the tail base and genital area, can be distinguished from UD by lesion distribution and appearance (Percy and Barthold 2007). Fighting wounds normally heal rapidly as opposed to the more chronic course typical of UD. In the research setting experimental

interventions must also be taken into consideration. This includes genetic alterations such as those that affect the skin barrier or immune function that could be interpreted as UD lesions. Mutant phenotypes are an especially important consideration given that the B6 is an exceedingly common "background" strain for generating genetically engineered mice (Lawson et al. 2005). Additionally, UD is characterized as poorly responsive to treatment which has been proposed as a diagnostic feature (Kastenmayer et al. 2006). However, no standardized treatment exists which calls into question the usefulness of response to therapy as a diagnostic criterion. Biopsy and bacteriology are occasionally performed to rule out other causes of dermatitis. Bacteriology typically reveals superficial colonization by normal constituents of the epidermal microbiota, particularly staphylococcal organisms (Andrews et al. 1994, Kastenmayer et al. 2006, Krugner-Higby et al. 2012). Bacterial infection may be complicating factor, however antibiotic treatment of mice with UD rarely results in significant clinical improvement (Andrews et al. 1994, Kastenmayer et al. 2006). Typical histopathology is described in the subsequent section.

Histopathology of UD lesions

As its name would suggest, the hallmark lesion of UD is a multifocal to coalescing ulcerative dermatitis. The ulcerations commonly have an associated serocellular and neutrophilic crust. The dermatitis is characterized by a mixed infiltrate predominated by neutrophils and mast cells with variable numbers of lymphocytes, macrophages, and plasma cells. The inflammation in the dermis can be severe, effacing normal architecture, and may extend into the subcutis. The skin adjacent to the ulcers is frequently hyperplastic with para- and orthokeratotic hyperkeratosis (Andrews et al. 1994, Hampton et al. 2012, Kastenmayer et al. 2006, Kimura 2012, Kruger-Higby et al. 2012, Williams-Fritze et al. 2011). Cystic hair follicles may be seen in lesional skin,

but a perifollicular pattern of dermatitis is not considered typical of UD (Duarte-Vogel and Lawson 2011, Hampton et al. 2012, Williams et al. 2012). Granulation tissue and dermal fibrosis may also be present depending on the chronicity of the lesion (Andrews et al. 1994, Kimura 2012, Williams-Fritze et al. 2011).

Proposed etiologies

Since the initial description of UD many proposed etiologies have been suggested. In 1994, it was suggested that the characteristic ulcerations were secondary to immune complex vasculitis. Andrews et al. described IgG, IgM, and fibrinogen deposition in dermal vessels of UD affected B6 mice using immunofluorescence. No such deposits were observed in unaffected mice. However, murine models with immune complex vasculitis do not exhibit an ulcerative dermatitis like phenotype (Sundberg et al. 2011). Furthermore, in several subsequent studies on UD in which histology was performed vascular lesions were not noted (Hampton et al. 2012, Kastenmeyer et al. 2006, Williams-Fritze et al. 2011).

Oxidative stress leading to pruritus and secondary ulceration has been suggested as a potential cause of UD. In a study describing vitamin E as treatment for UD the authors remarked (Lawson et al. 2005),

> "Because the one clinically uniting factor among all cases of UD is pruritus followed by self-mutilation and because the dermis contains large amounts of lipids, the positive therapeutic response to anti-oxidants- regardless of source- suggests that oxidative injury is a component of the cause or maintenance of UD lesions."

Despite this assertion several subsequent studies have failed to support his connection between UD and oxidative injury. On such study evaluated vitamin E supplementation as a way to prevent UD and instead found that a vitamin E fortified diet was associated with an increased

risk of UD (Mader et al. 2010). In addition, oxidative stress pathways of UD affected B6 mice, as assessed by gene transcription, were found to be comparable with normal wound healing profiles suggesting that oxidative stress response is not impaired (Williams et al. 2012).

UD has also been speculated to be caused by a behavioral disorder. In one study a serotonin promoting diet (originally intended to reduce barbering behavior as a model of trichotillomania) increased the risk of UD (Dufour et al. 2010). The authors also noted that pretreatment scratching behavior was predictive of UD development. The increased frequency in females and development of the UD "as the mice mature reproductively" were cited as also being consistent with human skin picking behavioral disorders. These findings, along with the absence of a consistently described primary dermatologic lesion, have been interpreted as evidence that abnormal repetitive scratching behavior is the driving force behind the ulceration characteristic of the condition (Dufour et al. 2010, Williams et al. 2012). It is noteworthy that, in addition to its well-known role as a neurotransmitter, serotonin is a potent pruritogen when injected into the dermis of mice (Akiyama et al. 2010, Yamaguchi et al. 1999) and it is unknown whether a serotonin promoting diet could sufficiently increase peripheral serotonin concentrations to incite clinically relevant itch. Psychogenic causes of itch are considered a diagnosis of exclusion in human medicine (Garibyan et al. 2013a). Similarly it seems prudent to carefully consider other systemic, metabolic, and neuropathic causes of itch and pruritus before concluding that UD is behavioral in origin.

In 2011 Sundberg et al., described a form of follicular dysplasia affecting C57BL/6J mice that histologically resembles a human condition called central centrifugal cicatricial alopecia (CCCA). The authors also found that the four B6 substrains tested (J, NCr, Tac, and Crl) have a

hypomorphic form of alcohol dehydrogenase 4 which could impair the ability to remove excess retinol from the skin, thus leading to the follicular dysplasia. The CCCA-like condition in this cohort of B6/J mice has important clinical differences compared to classically described UD. Firstly, the CCCA-like follicular dysplasia was described in relatively young mice at 6-15 weeks of age. Secondly, alopecia generally preceded the development of ulcers. In contrast, the age of onset for UD is typically between 1-2 years of age and the ulcers are not consistently preceded by or associated with alopecia (Hampton et al. 2012, Williams et al. 2012). Furthermore, a perifollicular distribution and follicular dysplasia have not been observed in subsequent studies of UD involving histopathology (Duarte-Vogel and Lawson 2011, Hampton et al. 2012). As a result it has been suggested that the CCCA-like condition in young C57BL/6J mice represents a separate entity from the UD syndrome that affects older B6 mice (Duarte-Vogel and Lawson 2011, Hampton et al. 2012, Williams et al. 2012).

Some contributory factors including diet, bacterial infection, and season have been implicated in the pathogenesis of UD. Caloric restriction has been reported to reduce the incidence of UD relative to ad lib fed mice (Blackwell et al. 1995, Pugh et al. 1999, Sell et al. 2000, Turturro et al. 2002). For instance, in a study by Blackwell et al. 26% of female mice on an ad lib diet developed UD during their lifetime while only 2% of the calorically restricted female mice developed UD. Interestingly it has been reported that insulin receptor substrate (Irs) 1 knock out mice are completely resistant to UD compared to wild type B6 controls (Neuhaus et al. 2012). Calorie restricted mice and Irs-1 deficient mutants both have a prolonged lifespan. As a disease of aged mice, it is unclear whether the reduction in UD is related to this delayed senescence phenotype in these mouse models or if there is a more direct link between metabolism and UD. Bacterial infection of UD wounds have largely been regarded as

opportunistic secondary invaders rather than pathogenic microbes. *Staphylococcus xylosus* and less frequently *S. epidermidis, Enterococcus* species, and *Bacillus* species are frequently isolated from UD lesions but these can also be found on the skin of clinically normal mice (Andrews et al. 1994, Kastenmayer et al. 2006, Kruger-Higby et al. 2012, Nagase et al. 2002, Tavakkol et al. 2010). Anecdotally, B6 mice in germ free conditions do not develop UD (audience member comment and panel discussion during conference session, unreferenced, 2013, Mouse Ulcerative Dermatitis- Perspective on a Persistent Problem, 64th AALAS National Meeting, Baltimore, MD). This could support a role for bacterial infection in the progression of UD. Alternatively, this observation may simply be a result of the technical challenges of maintaining mice in germ free conditions leading to relatively few aged B6 mice living in those conditions that would be at risk for UD. The prevalence of UD is also reported to vary by season (Kastenmayer et al. 2006, Sundberg et al. 1994). This has led to speculation that there may be an environmental allergy or humidity factor involved (Fox et al. 2007, Sundberg et al. 2011).

Treatment

Although the treatment of UD is typically unrewarding, many different types of therapy have been attempted (Andrews et al. 1994, Kastenmayer et al. 2006). They generally fall into one of three categories: topical treatments, systemic treatments, and environmental modification.

My conversations with visiting veterinarians from other institutions (Oregon Health and Science University, Memorial Sloan Kettering Cancer Center, The Rockefeller University, and Weill Cornell Medical College) and colleagues at national conferences suggest that many facilities use some form of topical treatment as their first line therapy for UD. Commonly this is an antimicrobial ointment or wound cream. Examples include triple antibiotic ointment, with or

without lidocaine, and silver sulfadiazine. Several additional topical treatments are described in abstracts and posters presented at conferences including camphor/pramoxine/zinc lotion (Crowley et al. 2008) and cyclosporine (Feldman et al. 2006). Topical corticosteroids have also been described in the treatment of UD (Andrews et al. 1994, Mader et al. 2010), but their usefulness is limited in the laboratory setting due to the confounding effects of steroids on many types of research.

Systemic treatments, similar to the topical treatments, generally involve antibiotics and analgesics. Specific analgesics that have been reported include ibuprofen (Ezell et al. 2012) and acetaminophen (Kastenmayer et al. 2006). In addition, vitamin E and fatty acid supplementation have been suggested for their antioxidant capacity and to improve general skin health, respectively (Lawson et al. 2005, Mader et al. 2010). One unique therapy that has been described to reduce scratching and promote lesion healing is maropitant citrate (Williams-Fritze et al. 2011). As a neurokinin type 1 (NK1) receptor antagonist maropitant citrate blocks the binding of substance P to NK1 receptors thus interfering with itch sensation.

Several nonpharmaceutical approaches have also been used. Bandaging (both of the lesions and the feet) has been attempted to reduce damage from scratching but this approach is technically challenging and time-intensive (Andrews et al. 1994). Environmental enrichment and delayed weaning have also been proposed to address a potential underlying behavioral component (Myers 1996, Williams-Fritze et al. 2011). In my communications, nail trimming, typically in conjunction with a topical antimicrobial, is a standard treatment at many facilities. Similar to bandaging, nail trimming reduces the amount of trauma that can be inflicted by scratching (Mufford and Richardson 2009, Seta 2009).

With all of these therapies, variable response to treatment and eventual relapse are typical making the management of UD unrewarding (Andrews et al. 1994, Kastenmayer et al. 2006). Given the gaps of knowledge regarding the etiology and pathogenesis of this condition identifying more effective treatments has proven incredibly challenging. Further complicating the issue is the need to balance clinical goals with research objectives. The treatment options may be limited by investigators' needs and must be practical in the context of a "herd health" type of animal care program. Therefore, significant challenges exist in identifying and employing interventions that will improve clinical outcomes and minimize the impact of this disease in research.

<u>Pruritus and scratching behavior in mice</u>

Processing of itch sensation

Itch is classically defined as "an unpleasant cutaneous sensation which provokes the desire to scratch" (Rothman 1941). Like the sensation of pain, itch sensation alerts an organism to the presence of a damaging stimulus in their environment, for example irritating plant fibers, and prompts a protective scratching response. Chronic itch is a feature of certain diseases, for example atopic dermatitis in both humans and veterinary species. In human medicine, it has been found that chronic itch can impact the quality of life of affected individuals comparable to chronic pain (Kini et al. 2011). In the absence of evidence to contrary, chronic itch in animals may be assumed to be similarly distressing. In regards to UD, pruritus is a prominent feature of the condition and it has been proposed that trauma from scratching is a major driving force behind the progression of UD lesions (Dufour et al. 2010, Hampton et al. 2012, Williams et al.

2012). Therefore, understanding the mechanisms of itch, or pruritus, has important implications for UD pathogenesis.

Recently there have been significant advances in understanding the neural processing of itch sensation using mouse models (reviewed in Akiyama and Carstens 2013, Garibyan 2013b, and Potenzieri and Undem 2013). The sensation of itch begins with the detection of one or more pruritogens. Pruritogens can be exogenous, such as the antimalarial drug chloroquine which is commonly injected intradermally to induce histamine independent itch in experimental models (Green et al. 2006). More importantly in the context of pruritic disease, a wide array of endogenous substances can act as pruritogens. In addition to the classic example of histamine, other endogenous substances that have been shown to elicit scratching responses in mice include proteases such as tryptase (Ui et al. 2006), neuropeptides such as substance P (Andoh et al. 1998) and endothelin-1 (Gomes et al. 2012), cytokines like IL-31 (Grimstad et al. 2009), and lipid mediators like leukotriene B4 (Andoh et al. 2011).

Pruritogens are sensed by unmyelinated C-fiber and thinly myelinated Aδ-fiber sensory afferents that respond to pruritogenic stimuli. These fibers activate neurons in the dorsal root ganglia (DRG) of the spinal cord (Akiyama and Carstens 2013). Neurons expressing Mas-related G-protein-coupled receptors (Mrgpr) A3 and D are important for processing itch, as are several neuron associated ion channels in the transient receptor potential (TRP) family, specifically TRP ankyrin 1 and TRP vanilloid 1 (Han et al. 2013, Liu et al. 2009, Liu et al. 2013). Neurons within the DRG use neurotransmitters such as gastrin-releasing peptide, substance P, and glutamate to transmit pruritic signals (Akiyama and Carstens 2013). This signal is then carried to the brain to initiate appropriate motor responses. Modulation of this signal can occur peripherally or centrally

resulting in inhibition or sensitization, depending on the signals received. Similar to what is seen with pain sensitization certain insults can result in enhanced pruritus to mildly pruritic stimuli (hyperknesis) or pruritus in response to normally nonpruritic stimuli (alloknesis) (Akiyama et al. 2012).

Clinical classification of itch

There are several clinical classification schemes for itch in human medicine. When categorized by underlying etiology chronic pruritus can be classified as dermatological, systemic, neurological, psychiatric/psychosomatic, mixed, or other (also known as "pruritus of unknown origin") (Ständer et al. 2007, Twycross et al. 2003). As indicated by the existence of "mixed" and "other" categories, the underlying etiology for pruritus may involve a complex combination of factors and a cause cannot always be identified. However, this classification system may be helpful in assessing potential underlying mechanisms for the pruritus classically associated with UD.

Dermatologic itch occurs when diseased or damaged skin incites pruritus (Ständer et al. 2007). During the course of dermatological disease pruritogens may be released from degranulated mast cells (histamine, substance P, endothelin-1, and tryptase), activated T cells (IL-31), or keratinocytes (leukotriene B4 and endothelin-1) (Dillon et al. 2004, Gomes et al. 2012, Kawakami et al. 2009). In human medicine some causes of pruritus in this category include atopic dermatitis, xerosis, cutaneous drug reactions, and cutaneous T-cell lymphoma (Ständer et al. 2007). Typically dermatologic causes of pruritus are associated with primary skin lesions such as wheal/flare reactions or dry skin, which may be confounded by secondary lesions from scratching (Ständer et al. 2007).

Chronic pruritus can also occur in response to neurological dysfunction (Ständer et al. 2007, Garibyan et al. 2013b). Nerve damage, compression, or irritation may initiate the sensation of itch. Unless the neuropathy was caused by trauma that resulted in scarring, the skin will generally appear clinical normal or have only secondary lesions caused by scratching (Ständer et al. 2007). Small fiber neuropathy, post-herpetic neuralgia, and brachioradial pruritus are a few examples seen in human medicine (Garibyan et al. 2013b, Oaklander 2012, Stumpf and Ständer 2013). In this setting patients often describe symptoms of overlapping pain and pruritus that may feel like electrical shock or "pins and needles" (Stumpf and Ständer 2013). Localization of pruritus to a specific dermatome, or a discrete area of skin innervated by a specific nerve-root, is also consistent with neuropathic itch (Oaklander 2012). Small fiber neuropathies which can cause pruritus may be diagnosed by demonstrating decreased epidermal nerve fiber density in a skin biopsy using immunohistochemical staining to target protein gene product 9.5 (Oaklander 2012, Stumpf and Ständer 2013). Imaging with modalities such as MRI may help identify areas of nerve compression that can cause pathologic itch (Oaklander 2012, Stumpf and Ständer 2013).

Systemic diseases, including chronic renal failure, liver disease, hematological diseases, and visceral neoplasms, have also been documented to cause chronic pruritus in human medicine (Garibyan et al. 2013b, Ständer et al. 2007, Twycross et al. 2003). The precise pathogenesis of itch in many systemic conditions is incompletely understood. It is suspected that a combination of dysregulated inflammation and inappropriate opioidergic signaling activate itch sensing pathways to result in pruritus (Garibyan et al. 2013b, Twycross et al. 2003). Also many of these conditions may alter skin physiology, further predisposing affected persons to pruritus (Ständer et al. 2007). Diagnosis of a systemic disease recognized to provoke itch (cholestatic liver disease,

uremic renal disease, or certain cancers) is typically achieved using standard clinical pathology and imaging modalities (Garibyan et al. 2013a).

Psychogenic or psychosomatic itch is recognized as an entity in human medicine (Garibyan et al. 2013b, Ständer et al. 2007). However, it is considered to be a diagnosis of exclusion and requires rigorous testing to rule out the previously described dermatologic, neuropathic, and systemic diseases that could cause itch by other pathophysiologic mechanisms (Garibyan et al. 2013a). Psychogenic itch has been associated with depression, obsessive-compulsive disorder, anxiety, and substance abuse disorders in human medicine (Garibyan et al. 2013b, Ständer et al. 2007). The mechanisms by which these conditions incite pruritus are poorly understood (Garibyan et al. 2013b).

Regarding "mixed" and "other" categories of itch, as the field of itch sensation and pruritic diseases continues to advance it is becoming apparent that pruritus is often multifactorial. For instance, a person with renal dysfunction may have dermatitis related to dry skin as well as uremic factors influencing their perception of itch sensation (Garibyan et al. 2013a, Ständer et al. 2007). Examples of "mixed" causes of pruritus serve as a reminder to refrain from oversimplifying pruritus pathways in our quest to understand UD pathogenesis and develop treatments for pruritic conditions.

The clinical classification scheme described above is not frequently applied in veterinary medicine but it may be a reasonable tool for assessing the proposed etiologies of pruritus as it applies to UD. Dermatologic causes of itch have been suspected for UD including bacterial pyoderma, follicular dysplasia, or allergic disease (Kastenmayer et al. 2006, Kruger-Higby et al. 2012, Sundberg et al. 2011). However, a consistently documented primary dermatologic lesion

has remained elusive (Dufour et al. 2010, Williams et al. 2012). Mice with UD frequently

manifest with significant secondary lesions, such as excoriations and ulceration, early in the

clinical course of disease. It is unclear whether this is a consequence of the rapid progression of

UD which may obscure primary lesions or whether it represents evidence against a

dermatological cause of UD. Systemic disease has been considered in several studies in which

full necropsies were performed on UD affected mice but systemic disease was not found to be

associated with UD (Andrews et al. 1994, Lawson et al. 2005). A psychogenic cause, particularly

a compulsive scratching disorder, has been suggested as the underlying etiology of UD (Dufour

et al. 2010). As a diagnosis of exclusion, there is much more to learn about UD before this

compulsive disorder hypothesis can be adequately tested. Neuropathic causes of itch in UD have

likely been under explored. A neuropathic mechanism would result in intense scratching

behavior in the absence of a primary dermatologic lesion, similar to what has been observed

clinically for mice with UD. There is an opportunity for future UD research to assess indicators

of neuropathic changes such as abnormal intraepidermal nerve fiber density.

Mouse strain and scratching responses

UD is associated with B6 mice and closely related inbred strains and genetically

engineered mice on a those backgrounds. Therefore, it is presumed that at least some of the

factors that predispose mice to UD are heritable. Although no such research is available, direct

heritability studies could provide important insights into the genes that influence UD

development. By crossing B6 mice with another well-characterized strain not predisposed to UD

(such as BALB/c or DBA/2) and observing which genes are associated with the disease in their

hybrid offspring it may be possible to identify genes that are important for UD development.

Unfortunately the late disease onset would require a substantial investment in time and resources

to conduct these studies, which may be prohibitive for many researchers. However, the uniquely

abundant genetic resources for mice, especially the B6 mouse, could greatly facilitate

investigations. For instance, the existence of recombinant inbred strains and thorough SNP

mapping of common mouse strains are powerful tools for interrogating genetic influences of a

heritable disease that are unavailable for most veterinary species.

Although no literature was found that examined UD heritability directly, two studies have

evaluated the response of different mouse strains to pruritogens (Green et al. 2006, Inagaki et al.

2001). In both studies scratching responses following injection of pruritogens such as histamine

and chloroquine were significantly different between mouse strains. Inagaki et al. found that ICR

mice (an outbred mouse stock) had a profound scratching response to histamine while a few

other mice including the B6 and the two WBBFF1 hybrids tested had more modest but

statistically significant increases in scratching. Green et al. also observed a relatively robust

scratching response to histamine in B6 mice and another inbred strain, AKR. However, more

importantly they characterized an inverted U-shape dose response curve to chloroquine injection.

The authors of this study suggests that other studies, such as the one by Inagaki et al., that only

used a single dose may be challenging to interpret accurately. Regarding B6 mice specifically,

Green et al. it was noted that,

> "Using only a low dose (e.g., 200 μg), one would conclude that the C57BL/6
> strain was among the most responsive to chloroquine of the 11 strains tested.
> Once full dose-response information is collected, however, it becomes clear that
> this strain shows high potency but low efficacy, and by the latter criterion is
> among the least responsive overall."

In addition, they found an inverse correlation between thermal nociception and pruritogen-

induced scratching. Strains that have been previously shown to be sensitive to thermal

nociception (as characterized by low 49 °C tail-withdrawal latencies) were relatively resistant to

chloroquine induced itch suggesting that the genetic mechanisms for thermal nociception and itch may be related. These studies provide evidence that scratching behavior, at least under some conditions, is strain related. Taking this concept a step further, it is possible that UD of B6 mice may be related to a strain specific tendency for abnormal scratching responses to pruritogenic stimuli compared to other commonly used inbred strains.

Summary

In reviewing the available literature it is clear that there are major gaps in our understanding of UD. While the clinical syndrome of pruritic older B6 mice with rapidly progressive ulcerative skin lesions is well recognized, many epidemiological factors are poorly described or controversial. Similarly the various treatments used and their relative efficacy is not apparent from this traditional literature review. In order to address some of these concerns, this thesis includes a systematic literature review of risk factors and treatments for UD. The goal of this aspect of the project is to assess the quality of evidence for the risk factors that appear in the literature, specifically age, sex, diet, and season. Also the review will assess the quality of evidence for various reported UD treatments.

While early investigations into UD focused on the possibility of a primary dermatopathology (e.g. vasculitis of cutaneous vessels or follicular dysplasia) several recent studies have advocated scratching behavior as a primary driver for UD. Also, as only a few closely related strains of mice develop UD, the condition appears to have a genetic component. One potential explanation that combines these two features is that B6 mice have a predisposition to excessive or abnormal scratching behavior. In order to test this hypothesis, an experiment was conducted which compared pruritic responses of B6, BALB/c, DBA/2, and ICR under several

conditions to evaluate whether B6 mice showed increased frequency or duration of scratching behavior.

Chapter 2: A systematic review of risk factors and treatments of ulcerative dermatitis of C57BL/6 mice

Introduction

Ulcerative dermatitis (UD) is a common condition of certain strains of laboratory mice, especially C57BL/6 (B6) mice. The condition is characterized by intense scratching and ulcerative skin lesions of the dorsal cervicothoracic region that are notoriously resistant to treatment (Andrews et al. 1994, Kastenmayer et al. 2006, Lawson et al. 2005). Large numbers of mice are affected with UD each year as B6 mice and genetically engineered mice on a B6 background are some of the most commonly used mice in research (Bryant 2011, Flurkey et al. 2006, Lawson et al. 2005). Although an overall UD prevalence or incidence is unknown for B6 mice, in some reports over 30% of the mice developed UD during the study period (Andrews et al. 1994, Hampton et al. 2012). Mice with this disease experience distress related to the severe pruritus and the progressive nature of the lesions (Andrews et al. 1994, Dufour et al. 2010, Hampton et al. 2012). Welfare concerns and the potential confounding effects of this disease on research endpoints frequently lead to euthanasia of affected mice (Lawson et al. 2005). However, despite the devastating impact of this condition in laboratory animal medicine the pathogenesis of UD is poorly understood and, accordingly, a consistently effective treatment is unavailable.

The cause of UD is speculated to be multifactorial (Duarte-Vogel and Lawson 2011, Williams et al. 2012). It has been reported that the risk of UD is affected by sex, age, season, and certain diets although not all of these effects have been consistently observed (Kastenmayer et al. 2006, Sundberg et al. 2011, Williams-Fritze et al. 2011). For instance, female mice have been found to be at increased risk in some reports (Kastenmayer et al. 2006, Sundberg et al. 1994) but

not in others (Andrews et al. 1994, Lawson et al. 2005, Mader et al. 2010). In addition to being clinically useful, identifying reliable risk factors may be advantageous in forming hypotheses regarding the etiology of UD. Therefore, the first aim of this systematic review is to identify peer reviewed literature that compares incidence or prevalence of spontaneous UD by sex, age, season, or diet and to evaluate the scientific evidence for these risk factors.

In addition, various treatments for UD have been reported with limited success (Andrews et al. 1994, Ezell et al. 2012, Lawson et al. 2005, Williams-Fritze et al. 2011). As a result, the treatment of UD is largely determined by clinician preference and personal experience. The second aim of this review is to identify studies that report treatments for UD and the quality of evidence supporting the use of the treatment.

Materials and methods

Search strategy

PubMed, PubMed Central, and Google Scholar databases were searched using the terms "ulcerative dermatitis" AND "C57BL/6." The searches were performed between December 2013 and January 2014. Abstracts for the articles were screened for relevance and full text versions of relevant articles were obtained for further review. No date restrictions were used in any of the searches.

Eligibility and exclusion criteria

For inclusion in this review, articles had to meet the following criteria:

1. Compare the risk of spontaneous UD in wild type C57BL/6 mice by sex, season, diet, or age OR compare healing or resolution of UD lesions by intervention
2. Measurements of criteria 1 are stated in the report
3. Original research paper
4. Published in a peer-reviewed section of the journal

5. Full text available in English

Reports describing mite-associated ulcerative skin lesions were excluded from review. In addition, descriptions of ulcerative skin lesions of genetically engineered or mutant mice were not reviewed.

Quality analysis - risk factors

For articles reporting potential risk factors for UD information was collected regarding study type, blinding, the number of animals per study group, definition of UD, method of UD diagnosis, and whether or not the aim of the study was to assess UD risk factors. Study type was categorized as previously described (Swann and Skelly 2013):

A: Blinded randomized controlled trial comparing 2 interventions
B: Controlled trial lacking either blinding or randomization
C: Prospective cohort study
D: Prospective case–control study
E: Retrospective cohort or case–control study
F: Prospective study with single intervention
G: Retrospective case series with single intervention

The definition given for UD was classified as follows (adapted from Wylie et al. 2012):

Not defined: Only the words "ulcerative dermatitis" are used
Partially defined: Brief description of ulcers with scratching or pruritus
Well defined: Complete description of lesions and location in mice with appropriate signalment

Similarly, whether the aim of the paper was to assess UD risk factors was scored as follows (previously described in Wylie et al. 2012):

No: The aim of the paper was unrelated to epidemiologic aspects of UD
Partially: The study was an epidemiological study into aspects of UD
Yes: The primary aim of the paper was to identify risk factors

Quality analysis – treatments

Reports of treatments or interventions for UD were analyzed using previously published guidelines for methodological quality (SIGN 2014). Information regarding study type, number of animals per study group, randomization, blinding, intervention studied, enrollment criteria, exclusion criteria, and outcomes were tabulated. Outcome measures of particular interest included percent reduction of lesion size and percentage of animals with complete resolution.

Reporting of results

Variation in outcome reporting between studies precluded quantitative analysis. Information regarding study design and the reported findings were compiled into tables.

Results

Search results

Initial database searches identified 318 articles. After removing duplicate publications the total number of reports for abstract review was 233. Abstract level review revealed 28 papers that met the inclusion criteria. Most reports were ineligible for more than one criteria (therefore the following numbers add up to >205), but the most frequently encountered cause for exclusion were publications that did not compare risk factors or treatment outcomes of UD (n=199). Other common reasons for exclusion were: description of dermatitis in mutant or knock-out mice only (n=79), not an original research paper (n=50), and dermatitis that was experimentally induced (n=35). Of the remaining 28 articles reviewed, an additional 16 reports were excluded. Most of these exclusions were articles that, upon further review, did not compare incidence or treatments of spontaneous UD in wild type B6 mice. At this step of the literature search there were no articles comparing treatments that met all of the criteria, however three articles from peer

reviewed journals compared treatments among wild type B6 mice and genetically engineered

mice on a B6 background. These three articles comparing treatments and nine articles which

addressed potential risk factors were reviewed for content (see Figure 2.1).

<u>Risk factors</u>

Overview

Nine publications met the inclusion criteria for the assessment of UD risk factors, and

these are detailed in Table 1. Of these, none compared risk by season. Four of the articles

compared risk by diet group only. Two studies compared UD risk by sex and diet, and another

analyzed risk by sex, age, and diet. The final two studies evaluated UD risk by sex and age.

Diet as a risk factor

Of the studies reviewed for content, seven compared UD risk in mice receiving different

diets (summarized in Table 2.2). Caloric restriction, either by quantity restriction or feeding of a

calorie reduced diet, was associated with a lower risk of UD in 4 studies. Three of the four

studies showed a strong, significant effect of caloric restriction ($p < 0.005$). The fourth study,

Blackwell et al, did not report on the level of significance but a "notable" difference between ad

libitum fed groups (26% lifetime prevalence in females, 13.5% in males) and diet restricted

groups (2% for females, 0.3% for males) was described. Neuhaus et al. compared the risk of UD

in mice fed a high fat diet as compared to a normal control diet, as well as the effect of lithium

supplementation. The high fat diet group had a higher risk of UD but this did not reach statistical

significance ($p = 0.14$). Lithium supplementation significantly increased UD risk ($p < 0.01$).

Dufour et al. evaluated the use of a serotonin promoting diet for the treatment of barbering and

found that mice on the serotonin promoting diet had a significantly higher risk of UD. Kruger-Higby et al. compared the risk of UD in female mice fed either a semipurified diet or the same diet with added conjugated linoleic acid. The trial was of short duration (4 weeks) using relatively young mice (4 months of age) and none of the wild type B6 mice developed UD during the experiment.

Age as a risk factor

Three studies compared UD risk by age. Two of these studies used prospective cohorts sacrificed at predetermined time points and compared the rate of UD among these cohorts. Hampton et al. found a higher risk of UD in the cohort sacrificed at 19 months of age (26.7% for males, 35% for females) compared to the 13 month cohort (6% for mice of both sexes), but did not report a p value. In another prospective cohort study by Andrews et al. no clear trend was observed with 37% mice affected at necropsy at 17 months of age, 11% affected at 19 months, 0% at 21 months, and 20% at 23 months. In the same facility clinical cases presented to veterinary staff had an average age of onset of UD of 20 months. Turturro et al. presented a "Cumulative probability of death from dermatitis" graph and noted that UD occurred in animals between 550 days and approximately 730 days of age. It should be noted that mice with UD in this study were euthanized at the time of diagnosis due to poor prognosis.

Sex as a risk factor

Of the 5 studies that compared the risk of UD in female and male mice 3 studies reported an increased risk in female mice (Blackwell et al. 1995, Dufour et al. 2010, Turturro et al. 2002) while 2 studies reported a similar risk of UD for both sexes (Andrews et al. 1994, Hampton et al.

2012). Two of the studies that reported a higher rate of UD in female mice involved ad libitum feeding versus caloric restriction diet trials and both papers reported a significant effect of both diet and sex. The third study evaluated the effect of a serotonin promoting diet with supplemental tryptophan and an increased carbohydrate:protein ratio and found that the diet increased the risk of UD especially among female mice. Four of the nine studies used mice of only one sex (males only n=2, and female only n=2), so it is not possible to compare prevalence or incidence between the sexes for these reports.

Two articles compared age of UD onset by sex. Turturro et al. found that affected females were seen at younger ages than males, but the peak incidence for females was slightly later at 600-750 days of age as opposed to 550-700 days of age in males. In comparison, Hampton et al. observed that the mean age of onset did not differ by sex. However, all the mice in this study were sacrificed by 19 months of age, or approximately 570 days, so differences apparent after that time would not have been detected.

Quality analysis for studies identifying potential risk factors

The majority of the publications (7/9) described controlled trials with animals being grouped by dietary intervention. Two papers described prospective cohort studies of mice of different ages at the time of necropsy and tissue collection. Blinding was only described in one of the publications (Dufour et al. 2010). Minimum experimental group size ranged from 10 to 266 with a median of 25 mice per group.

The definition used for UD and the means by which it was diagnosed was highly variable among the studies examined (Table 2.1). In regards to a UD case definition, in two publications

there was no definition provided. In three publications a brief description was reported including ulcers and self-trauma. In four publications a thorough description of UD was provided. Techniques used to diagnose or confirm UD were similarly variable and included gross lesion character, physical exam by a veterinarian, histology, or, in some cases, no diagnostic criteria or confirmatory testing was reported.

Identifying risk factors for UD was the primary aim for only one of the studies analyzed. Another four publications were partially designed to assess epidemiological aspects of UD. The remaining four quantified the risk of UD in the different experimental groups although the aim of these studies was to assess the effects of caloric restriction.

Reported treatments and quality analysis

Three reports evaluated treatments for UD (Table 2.3). Ezell et al. compared the palatability and efficacy of two different formulations of ibuprofen in drinking water and concluded that the liquid-gel formulation was more effective. Lawson et al. evaluated resolution of UD wounds following vitamin E supplementation. The third study by Williams-Fritze et al. evaluated maropitant citrate as a potential treatment and found the 1 mg/kg dose to be significantly more effective than the 5 mg/kg dose and the saline control. In assessing the quality of evidence, two of the three lack a control group. In Ezell et al. all mice were given one of two ibuprofen formulations and there was no untreated or alternative therapy control group. The study of vitamin E treatment by Lawson et al. reports a rate of response to treatment and makes reference to historical recovery rates in other published reports. However, there was no untreated or alternative therapy control.

Discussion

Ulcerative dermatitis of B6 mice is one of the most common and frustrating conditions managed by laboratory animal veterinarians and animal care staff. Efforts to manage this disease are undermined by a lack of knowledge about its etiology and pathogenesis. Characterizing reliable risk factors can aid in the recognition of susceptible animals and provide clues regarding the etiology of UD.

Caloric restriction, via reduction in food quantity or the feeding of a lower calorie diet, was consistently associated with a decreased risk of UD in all of the publications that evaluated this dietary intervention. Caloric restriction has also been shown to increase lifespan in B6 mice (Blackwell et al. 1995, Pugh et al. 1999, Turturro et al. 2002, Weindruch and Walford1988). As a disease most commonly seen in aging mice, the protective effect of caloric restriction for UD may be related to these changes in aging phenotype and longevity. It is also possible that differences in gut microbiota associated with different diets may modulate inflammation or other aspects of the disease process. The mechanism by which caloric restriction reduces the incidence of UD has not been explored and remains a promising avenue for future research. One important caveat is that assessing UD incidence or prevalence was not the primary aim in any of the reviewed caloric restriction studies. As such, clear inclusion and exclusion criteria, case definitions, and confirmatory diagnostics were not described. Other dietary interventions including a serotonin promoting diet and a lithium supplemented diet also significantly altered UD risk. This evidence was limited to a single study in both cases, but these studies provide further evidence that diet may be an important factor in the development of UD.

There is conflicting evidence of an increased risk of UD in female mice. Two studies that met the inclusion criteria for review revealed no significant differences in UD risk for female and male mice (Andrews et al. 1994, Hampton et al. 2012). In contrast, three studies, all of which involved dietary interventions, showed a significantly increased risk for female mice (Blackwell et al. 1995, Dufour et al. 2010, Turturro et al. 2002). These studies also showed a significant effect of diet on UD risk. It is possible that certain risk factors, such as ad libitum feeding, may be more important in female mice. Sex specific differences in response to diet, especially caloric restriction, may be relevant to the pathogenesis of UD and merits further investigation.

Age and seasonality were also evaluated as risk factors for UD. For the three studies that examined the risk of UD by age, UD was most commonly seen between 14 and 24 months of age with some variation between the studies. Two of these studies (Andrews et al. 1994, Hampton et al. 2012) were cohort studies with predetermined terminal collection intervals. This methodology may underestimate the average age of onset of UD as animals that would have developed UD after those predetermined time points are not represented in the data. A seasonal pattern has been reported previously (Kastenmayer et al. 2006, Sundberg et al. 1994), but none of the publications that met the inclusion criteria of this review assessed this potential risk factor. Seasonality is an important diagnostic feature in dermatology and confirming the previously described seasonal pattern to UD could have substantial implications. Different seasons may be associated with different environmental risk factors such as allergens and humidity. However, seasonality should be interpreted with caution in the laboratory setting as other annual cyclic events such as funding cycles and the academic year may alter the demographics of mouse populations in the vivarium, which may create the appearance of a seasonal effect.

One limitation of this review is the small number of publications analyzed. Although 233 unique articles were identified by the search terms most mentioned UD only briefly as a cause of attrition among study animals. Few of these studies compared UD risk or treatments between groups of mice. The inclusion criteria limited the analysis to spontaneous UD in wild type B6, and descriptions of UD or UD-like lesions in mutant or genetically engineered mice were excluded. The exclusion of genetically engineered mice (GEM) was intended to omit reports of GEM that have lesions that appear grossly similar but have a differing underlying pathogenesis than UD. Certain GEM strains are known for developing open skin lesions that may be called "ulcerative dermatitis." For instance E- and P-selectin deficient mice develop conjunctivitis and submandibular ulcerative skin lesions which appear to be T cell dependent (Forlow et al. 2002). Another mutant strain, a stearoyl CoA desaturase deficient mouse, develops ulcerative skin lesions. This strain has been suggested as a model for UD although the mutant phenotype also includes an absence of sebaceous glands which could interfere with evaluating a condition with dermatologic manifestations (Krugner-Higby et al. 2012). It is entirely possible, if not likely, that at least one GEM that develops ulcerative skin lesions as part of its mutant phenotype has a similar underlying pathological mechanism to the B6 UD syndrome and could be a valid model. An opportunity for future study would be to compare mutant mouse strains with an ulcerative dermatitis-like phenotype to spontaneous UD in wild type B6 mice to identify those that are highly similar. GEM with spontaneous ulcerative skin lesions with a similar distribution and character to the B6 UD syndrome may provide hints about the genetic mechanisms that influence UD.

The publications that analyzed risk factors used several different substrains of B6 mice including C57BL/6J (Dufour et al. 2010), C57BL/6NNia (Andrews et al. 1994, Sell et al. 2000,

Turturro et al. 2002), C57BL/6Crl (Hampton et al. 2012, Pugh et al. 1999), and B6 mice obtained

from Harlan (possibly substrain C57BL/6OlaHsd, Krugner-Higby et al. 2012). In addition, two

papers did not report the substrain used (Blackwell et al. 1995, Neuhaus et al. 2012). Substrains

of B6 have limited genetic differences, but if these differences modify the risk of UD or how UD

manifests then the combining of different substrains for analysis would be another limitation of

this review.

Availability of a full text English version of the article was selected as an inclusion

criterion to facilitate content review of the articles based on the primary language of the authors

of this report. The only article that failed to meet this criterion was a thesis in German entitled

"PCR-based investigation of the presence of herpesvirus in the peripheral vestibular system in

cats and dogs" (Parzefall 2010). Based on the translated abstract this research was unrelated to

risk factors for or treatments of UD. Furthermore, it was not published in a peer reviewed

journal. However, it is possible that research relevant to UD risk factors or treatments published

in languages other than English were not detected by the English language search terms.

Therefore, there may be other studies regarding UD that were not identified in this review. A

literature search involving other languages was beyond the scope of this project but could be

undertaken by colleagues with fluency in other languages to add to our understanding of this

condition.

One commonly cited paper on the epidemiology of UD did not meet the inclusion criteria

of this review, but merits brief mention. Kastenmayer et al. analyzed the prevalence of UD

among B6 and B6 background mice in a two year retrospective study. Significant findings

included an increased risk of UD in females, inducible nitric oxide synthase (iNOS) deficient

mice, and during the summer. This study was excluded from analysis as it combined mutant and wild type B6 mice for the measures of interest. As a retrospective study, there are certain limitations as the sex, age, and genotypes represented are determined by investigator needs meaning certain subsets may be underrepresented, for example geriatric male mice. However, the high incidence of UD in iNOS deficient mice is an interesting finding that has yet to be explored.

Various treatments for UD have been reported including caladryl lotion, cyclosporine, nail trimming, and bandaging (Andrews et al. 1994, Crowley et al. 2008, Feldman et al. 2006, Seta 2009). Unfortunately few treatments have been evaluated in a rigorous or controlled fashion. Many commonly cited treatments, such as nail trimming, can be traced to conference posters, abstracts, and anecdotal reports. In this review very few peer reviewed studies evaluating treatments could be identified. Specifically regarding the quality of evidence, there were studies that lacked a control group for comparison. Given the variability in UD incidence and recovery rates reported in the literature, the absence of controls raises serious concerns about determining the efficacy of a treatment. Several reports cited humane reasons for the lack of an untreated control group. Many institutions treat UD with a topical antimicrobial and/or analgesic and nail trimming with fair response, thus a "standard of care" treatment group could be a reasonable control option. There is a need for well-designed controlled peer-reviewed studies of UD treatments.

UD is a devastating disease that affects one of the most commonly used strains of mice in biomedical research. The impacts of UD on the research community such as confounding research endpoints, compromising animal welfare, and consuming animal care resources are

compounded by its rapidly progressive nature and poor response to treatment. While systemic reviews are relatively uncommon in veterinary medicine, they prove useful as a tool to summarize existing literature and can provide insight regarding areas where more research is needed. The results of this systematic review clearly show that more work is necessary to elucidate epidemiologic aspects of UD and to develop standardized diagnostic and clinical criteria to facilitate comparisons in future research. Additionally, this review has shown a paucity of controlled clinical trials comparing treatment options which are critical for developing an evidence-based approach to treating this common and refractory disease.

318 records identified

85 duplicates removed

233 records screened

205 abstracts excluded

28 full text articles screened for eligibility

19 full text articles excluded

12 articles included
9 risk factor related
3 treatment related

No publications on treatment met all criteria, after relaxing the criterion for excluding mutant mice 3 reports were identified
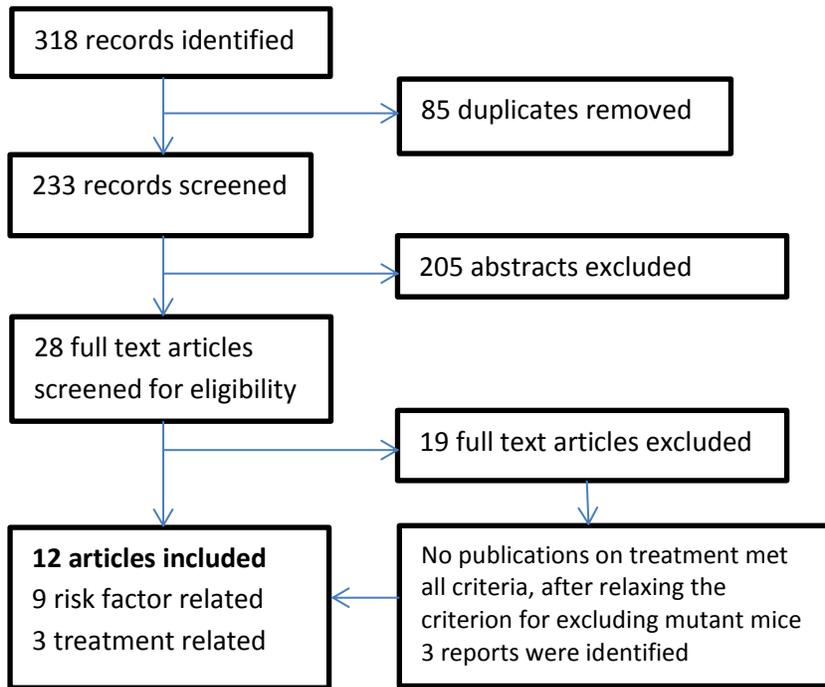
Figure 2.1. Flow chart of the search strategy used to identify articles for inclusion

Table 2.1. Characteristics of publications that compare UD risk by sex, diet, age, and season. Study type categories: A= Blinded randomized controlled trial comparing 2 interventions, B= Controlled trial lacking either blinding or randomization, C= Prospective cohort study, D= Prospective case–control study, E= Retrospective cohort or case–control study, F= Prospective study with single intervention, and G= Retrospective case series with single intervention.

	Study type	n	Blinding	UD definition	UD diagnosis confirmation	Aim to ID UD risk factors?	Comparison by:			
							Sex	Age	Diet	Season
Andrews et al. (1994)	C	18	Not described	Well described	Histology	Partially	X	X		
Blackwell et al. (1995)	B	266	Not described	Not defined	Unclear	No	X		X	
Dufour et al. (2010)	A	12	Yes (Experiment 1)	Well described	Identification by veterinarian	Partially	X		X	
Hampton et al. (2012)	C	25	Not described	Well described	Histology	Partially	X	X		
Krugner-Higby et al. (2012)	B	11	Not described	Well described	Histology, bacterial culture	Partially			X	
Neuhaus et al. (2012)	B	10	Not described	Partially defined: age & gross findings	Lesion character	Yes			X	
Pugh et al. (1999)	B	75	Not described	Partially defined: open wounds & excessive scratching	Unclear	No			X	
Sell et al. (2000)	B	31	Not described	Not defined	Lesion & response to treatment	No			X	
Turturro et al. (2002)	B	56	Not described	Partially defined: ulceration & self-mutilation	Unclear, possibly histology	No	X	X	X	

Table 2.2. Publications that compared UD risk by dietary intervention group. CR= caloric restriction, AL= ad libitum, HFD= High fat diet, SPD= serotonin promoting diet.

Paper	Dietary intervention	Control diet	Outcome	p value
Blackwell et al. (1995)	60% CR by quantity restriction	NIH-31 fed AL	Effect of diet and sex reported with UD risk highest in AL females and lowest in CR males	Not reported
Dufour et al. (2010)	Custom serotonin promoting diet (SPD)	Custom purified ingredient diet formulated to emulate standard rodent chow	Significantly higher risk of UD in SPD group	0.001
Krugner-Higby et al. (2012)	AIN76A with 1% conjugated linoleic acid added fed AL	AIN76A fed AL	No UD in any group of wild type B6	N/A
Neuhaus et al. (2012)	High fat diet (HFD), 35% fat fed AL	"Normal chow diet," 3.3% fat	Higher risk of UD in HFD group, but not significant	0.14
Neuhaus et al. (2012)	"Normal chow diet" with lithium chloride supplementation (1g/kg diet)	"Normal chow diet" with no added lithium	Significantly higher risk of UD in lithium supplemented group	<0.01
Pugh et al. (1999)	26% CR by feeding of a limited quantity of a reduced calorie diet starting at 1 year of age	Limited quantity of a higher calorie control diet	Significantly lower risk of UD in CR group	<0.005
Sell et al. (2000)	60% CR by AL feeding of reduced calorie diet	NIH-31 fed AL	Significantly lower risk of UD risk in CR group	<0.0001
Turturro et al. (2002)	60% CR by quantity restriction	NIH-31 fed AL	Significantly lower risk of UD in CR group	<0.001

Table 2.3. Characteristics of publications that compare healing or resolution of UD lesions by intervention. Study type categories: A= Blinded randomized controlled trial comparing 2 interventions, F= Prospective study with single intervention, and G= Retrospective case series with single intervention.

Paper	Ezell et al. (2012)	Lawson et al. (2005)	Williams-Fritze et al. (2011)
Study type	F*	F	A
N	14	71	30
Random-ization	Adequate	N/A	Adequate**
Blinding	Unclear	N/A	Adequate for pruritus scoring, unclear for lesion scoring
Intervention	Ibuprofen 1 mg/ml for 9 days in drinking water, either pediatric suspension or liquid-gel formula	NIH 31 diet + 3000 IU vitamin E for 8 weeks	Maropitant citrate IP 1 mg/kg or 5 mg/kg for 5 or 10 days
Enrollment criteria	B6 or B6 background, exam by veterinarian, defined as "single or multiple areas of the skin with excoriation, dermal ulceration with serocellular crusts or granulation and located on the dorsal scapular, dorsal thorarcolumbar, or lumbosacral regions."	B6 or B6 background; spontaneous UD lesion diagnosis by clinical appearance during routine health checks; not used in another study	Identified during routine health checks, veterinarian confirmed UD based on "physical exam and characteristic appearance of lesions"
Exclusion criteria	Severe UD: active bleeding, contracture, deep ulcers	None	Deep ulcers, other disease, iNOS deficiency
Lesion size reduction	65% reduction (liquid gel) vs 29% (suspension), mean reduction lesion size over 9 d	25% had 50-99% reduction, 9% had 12-30% reduction, 21% had no reduction in lesion size	10% reduction: 78% by day 15, 94% by day 57; 25% reduction: 59% by day 15 and 83% by day 43 (in 1 mg/kg group)
Complete resolution rate	Not reported (50-100% resolution rates available)	45%	Not reported
Other outcome measures	Food intake, scoring of pruritus, locomotor activity, and presence of grooming	Necropsy: Lymphadenopathy, splenomegaly	Pruritus score: days 29-50, 1 mg/kg group lower pruritus score
*No non-ibuprofen control			
**Randomized and sex matched, but no discussion of matching for genotype or age			

Chapter 3: Pruritic responses to epidermal injury in C57BL/6, DBA/2, BALB/c, and CD1 mice

Introduction

Ulcerative dermatitis (UD) is a condition characterized by pruritic open skin lesions in C57BL/6 inbred mice and related strains (Andrews et al. 1994, Kastenmayer et al. 2006, Sundberg et al. 1994). Affected animals are typically unsuitable research subjects and the lesions raise significant welfare concerns (Lawson et al. 2005, Mader et al. 2010). As a result many mice with UD are euthanized leading to significant economic losses and research interference. Although the cause is unknown there is evidence that self-inflicted trauma from hind limb scratching has an important role in the progression of UD (Dufour et al. 2010, Hampton et al. 2012, Percy and Barthold 2007).

B6 mice have been observed to have high rates of barbering, which has led to speculation that UD may be caused by abnormal grooming behavior in the strain (Dufour et al. 2010, Percy and Barthold 2007). Although barbering behavior does not appear to lead to UD (Dufour et al. 2010, Sundberg et al. 2011), recent findings have supported a role for scratching behavior in UD lesion progression (Dufour et al. 2010, Mufford and Richardson 2009, Seta 2009, Williams-Fritze et al. 2011). In a study by Dufour et al., a serotonin up-regulating diet was evaluated as a treatment for a barbering model of trichotillomania. Unexpectedly they found that mice receiving the test diet showed increased scratching behavior and had a higher incidence of UD. Furthermore, pre-treatment scratching was predictive of later UD development. In addition, treatments aimed at disrupting the itch-scratch cycle, such as substance P inhibition and nail trimming, have been reported to reduce UD lesion size and severity (Mufford and Richardson 2009, Seta 2009, Williams-Fritze et al. 2011).

Genetic factors have been shown to influence scratching behavior in response to exogenous pruritogens among various mouse strains (Green et al. 2006, Inagaki et al. 2001). A study by Inagaki et al. compared responses of different inbred and outbred mice to intradermal injection with serotonin and histamine. They found that histamine produced a profound increase in scratching frequency in ICR mice and a modest but significantly significant increase in B6 mice. Under the same conditions no significant increase in scratching was observed in most of the strains tested. Another study by Green et al. found that peak scratching occurred at a lower chloroquine dose in the B6 mouse compared to the other mouse strains. However, there is no comparison of spontaneous scratching behavior or scratching behavior in response to epidermal barrier injury between mouse strains in the literature. If B6 mice demonstrate more spontaneous scratching behavior or scratch more in response to mild epidermal insults, such as those that occur during routine grooming or handling for research, this could influence the development of UD.

The aim of this study is to assess whether B6 mice exhibit excessive scratching under resting conditions or when provoked by epidermal barrier disruption compared to DBA/2, BALB/c, and ICR mice. It is hypothesized that B6 mice will exhibit more spontaneous scratching behavior and that B6 mice will be more pruritic following mild epidermal barrier injury compared to the other mouse strains and one mouse stock.[1]

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[1] For the sake of simplicity, the genotypes of mice represented will be referred to collectively as strains in the remainder of this manuscript even though the ICR, as an outbred mouse, is technically a mouse stock.

Material and methods

<u>Mice</u>

Retired breeder female C57BL/6J (n=15), DBA/2J (n=10), and BALB/cByJ (n=10) mice were purchased from Jackson Laboratories (JAX West, Sacramento, California). Retired breeder female Crl:CD-1 mice (n=10) were obtained in-house from the Laboratory Animal Resources Center's foster and rederivation colony. There was some concern that the B6 mice might develop spontaneous UD during the study and need to be removed from the experimental group for health reasons. Due to those concerns 15 B6 mice were purchased so that there were 5 "replacement" B6 mice available. Mice were SPF for a wide range of common mouse pathogens as assessed by quarterly sentinel testing. Mice were kept on a 12:12-h light:dark cycle with a light period from 06:30 to 18:30. Mice were housed 4 per cage with one mouse of each strain/stock in each of the 10 cages to control for differences in microenvironment. They were housed in in standard individually ventilated mouse caging (One Cage, Lab Products, Seaford, Delaware) on paper based bedding (Biofresh, Absorption Corporation, Ferndale, Washington). Rodent chow (5053, Purina Test Diets, Saint Louis, Missouri) and water (Hydropac, Lab Products, Seaford, Delaware) were provided *ad libitum*. During the acclimation period most of the DBA/2 and BALB/c mice were noted to be thin, and supplemental gel diet (ClearH$_2$O, Portland, Maine) was provided to all cages every 2 weeks until the conclusion of the experiment. Nesting material (Nestlets, Anacare, Bellmore, New York) and wheels (Anacare, Bellmore, New York) were provided for enrichment. All animal procedures were approved by the Oregon State University IACUC and were performed in an AAALAC-accredited facility.

Scratching behavior

Mice were placed individually in 10.5 inch by 14.5 inch by 8.5 inch deep observation chambers. They were allowed to acclimate for 15 minutes and then their behavior was filmed for 1 hour using a video camera (Sony Corp., Tokyo, Japan) mounted on the ceiling of the room. To optimize lighting conditions while filming as close as possible to the active period of the mice, the first filming session started at 17:15 (PM) and then was repeated the next morning starting at 07:00 (AM). At the end of the morning behavior videotaping a spray test was performed as previously described (Dufour et al. 2010, Martin and Bateson 1993). Previously this test was demonstrated to be predictive of future UD development (Dufour et al. 2010). Briefly, each mouse was sprayed with a fine mist from water bottle two times. The subsequent behavior was filmed for an additional 15 minutes. The video was later reviewed and the number of scratching events was recorded. Mice often scratch, pause to lick their hind paw, and then continue scratching (Green et al. 2006, Wilson et al. 2013). As in previous studies of pruritus and scratching behavior in mice each scratching event was defined as ending when the hind paw was placed on the floor (Wilson et al. 2013). In addition, scratching duration was recorded as brief (<3 seconds) or long (≥3 s). This cut off was based on observations of scratching behavior during a pilot study (data not shown). The durations of long scratching events were recorded. Each mouse was filmed 6 times: baseline PM, baseline AM, baseline spray test (AM), 5 h post tape stripping injury (PM), 17 h post-injury (AM), and 17 h post-injury spray test (AM).

Tape stripping

Mice were anesthetized briefly with isoflurane. The hair on the right dorsum was removed with an initial clipping of the hair followed by the application of a depilatory cream

(Nair, Church & Dwight Co., Ewing, New Jersey). Then tape stripping was performed as previously described (Miyamoto et al. 2002, Onoue et al. 2009, Oyoshi et al. 2012). Briefly, a strip of cellophane tape (Scotch, 3M, Hutchinson, Minnesota) was adhered to the depilated skin and removed. This process was repeated 8 times using a new piece of tape each time. The mice were then recovered and returned to their home cage.

Tissue collection

Twenty four hours after the tape-stripping injury mice were euthanized by CO_2 exposure. The left dorsum was clipped. The entire dorsal pelt was then removed and sectioned for fixation in 10% buffered formalin. The long thin skin sections created where rolled from the cranial end to the caudal end around the wooden end of a cotton-tipped applicator prior to being placed in formalin. Following standard processing, 5 μm slices were stained with hematoxylin and eosin. The tape stripped tissue was evaluated for degree of barrier disruption, qualitative assessment of inflammation, inflammatory infiltrate depth, percentage of the surface area of the skin section that was affected, as well as number of mast cells per ten 400x fields. The overall severity of inflammation was scored semi-objectively using a five point scale (1-5, 5 = most severe) which was used to compare strains. Mast cell counts were performed in ten adjacent fields encompassing the most inflamed areas of dermatitis. The tape stripped skin was compared to skin samples from the contralateral side of the dorsum which had not been tape stripped.

Statistics

Comparisons within strains between different time points were performed using a paired t-test. ANOVA was used to look for significant differences in scratching frequency, severity of inflammation score, and mast cell counts amongst the four strains; and when they were present

an unpaired t-test was used for comparisons between B6 mice and the other three strains with correction for multiple comparisons. Scratching frequency was normally distributed and presented graphically as an average with error bars representing the standard deviation. Scratching duration for long bouts of scratching were skewed due to a small number of very long scratching events, therefore the data is represented as median duration.

Results

<u>Animals</u>

Shortly after arrival from the vendor seven DBA/2 mice and four B6 mice delivered litters of pups. In addition, there was a litter documented in cage #7 but the pups were cannibalized by post-natal day 2 before the dam could be positively identified. This unexpected complication was addressed with a 6 week acclimation period to give the mice time to wean their litters and recover from the metabolic demands of lactation prior to experimental procedures.

During the acclimation period two DBA/2 mice died. In both cases the remains were in poor condition (marked autolysis and partially cannibalized) so diagnostic necropsy was not pursued. No premonitory signs had been noted and no procedures had been performed on these animals prior to death. Replacement B6 mice were added to the cages to maintain a density of 4 mice per cage. These B6 mice underwent behavioral observations and tape stripping with the other mice in the cage. Two additional DBA/2 mice found dead without premonitory signs during the interval between baseline and epidermal barrier injury. Again the remains were in poor condition which precluded diagnosing a cause of death. These mice were also replaced with B6 mice to maintain the same cage density. However, since there were no corresponding baseline behavioral observations for these mice, they did not undergo tape stripping, post

epidermal injury behavioral observations, or tissue collection. All new social housing groups were allowed at least one week of acclimation prior to behavioral observations.

Due to the issues described above the final analysis includes data from 12 B6 mice, 10 BALB/c, 10 ICR, and 8 DBA/2 mice for baseline behavioral observations with 6 DBA/2 mice available for post-epidermal barrier disruption behavioral observations and tissue collection.

Scratching frequency at baseline and post injury

Scratching behavior was recorded prior to skin barrier disruption during two 60 minute sessions, one starting at 17:15 (PM) and one starting the next morning at 07:00 (AM). The ICR mice had significantly more scratching events recorded in the PM session compared to the AM (p = 0.004). No other mouse strain had a significant difference between baseline recording events. Under baseline conditions there was no evidence that B6 mice scratched more than the others. In fact, B6 mice had the least recorded scratching events of the four strains evaluated (Figure 3.1). They scratched significantly less than DBA/2 mice (p= 0.0068 PM and p= 0.0023 AM) at both time points, and significantly less than the BALB/c (p= 0.0042) and ICR (p= 0.00080) mice at the PM time point. The BALB/c mice scratched more than the B6 at the AM time point but the difference was not significant once after correcting for multiple comparisons (p= 0.021, corrected level of significance 0.017).

Following barrier disruption with tape stripping filming of scratching behavior was repeated at the same time points, with scratching 5 hours- and 17 hours-post epidermal injury being observed. At 5 and 17 hours post injury The BALB/c and DBA/2 showed no statistically significant increase in scratching behavior compared to baseline. There was a significant increase in scratching behavior following barrier disruption as compared to the baseline scratching

recorded during the same time period (i.e. PM baseline compared to 5 h post injury, and AM baseline compared to 17 h post injury) for both the B6 (p = 0.00037 and p= 0.0042) and ICR (p= 0.00032 and p= 0.0086). As with the baseline scratching behavior, the ICR showed a significant difference in scratching frequency at the PM and AM time points post epidermal barrier injury (p< 0.0001). Compared to the other strains, again there was no evidence that B6 mice scratched more frequently. As noted during baseline behavior recordings, B6 mice scratched the least. This lower frequency of scratching events was statistically significant compared to the ICR mice at the 5 h post injury time point (p= 0.0004). At 17 h post injury the B6 scratched significantly less than the DBA/2 mice (p= 0.0009).

Scratching duration at baseline and post injury

Short (<3 seconds) scratching bouts comprised the majority of scratching events under baseline conditions (Figure 3.2). The B6 mouse showed no evidence of having longer scratching events than the other strains. The B6 mice had the fewest long (≥ 3 seconds) scratching bouts with 11.5% (PM) to 4.3% (AM) being long duration scratching events. At 5 h post injury the percentage of short duration scratching bouts was remarkably similar amongst all 4 strains, ranging between 54.0% and 59.5%. By 17 h post injury the percentage of short duration scratching bouts was similar to baseline values, with the B6 mouse having the highest percentage of short scratching events at 89.6%. Long duration scratching events ranged from 3 to 39 seconds with a median of 5 seconds. The median duration of long scratching bouts is depicted in Figure 3.3. In examining maximum scratching duration by strain the B6 shortest maximum in the baseline testing while B6 and ICR mice had similarly short maximum scratching durations compared to DBA/2 and BALB/c mice (Figure 3.4).

<u>Spray test</u>

Scratching frequency at baseline and 17 h post injury time points is depicted in Figure 3.5. As in the previous analyses there is no evidence that the B6 mice scratched more frequently than mice of the three other strains tested, although they did scratch significantly less than the DBA/2 at baseline (p= 0.008) and 17 h post (p= 0.0009). In addition, the post injury scratching frequency was significantly different from baseline in the ICR (p= 0.0429). There was no significant difference in scratching frequency pre- and post-epidermal barrier injury in the B6, BALB/c, and DBA/2 (p >0.05). Regarding scratching duration, at baseline 53-60% of scratching events were of short duration for all strains (Figure 3.6). Post epidermal barrier injury the BALB/c had the largest proportion of long duration scratching events at 66.7%. The B6 did not show evidence have of having longer duration scratching events than other strains.

<u>Histology</u>

The tape stripped skin had gross lesions ranging from mild erythema to locally extensive dermatitis with associated crusts. Histology confirmed tape stripping successfully disrupted with epidermal barrier resulting in the removal of the outer most cornified layer of skin when compared to the control skin which had not been manipulated prior to tissue harvest. Consistently the tape stripped skin had suppurative dermatitis with multifocal to coalescing pustules (Figure 3.7). There was a range in regards to percentage of surface area affected by this pustular dermatitis (1% to 70%) and depth of inflammatory infiltrate (from very superficial to inflammation extending in to the subcutis). The overall inflammation severity score was not significantly different between strains (ANOVA, p= 0.256). Mast cells were observed at 11-60 per ten 400x fields with no significant differences by strain (ANOVA, p= 0.259).

Discussion

UD is a frustrating condition to manage and efforts to improve clinical outcomes have
been undermined by the poorly understood etiology of the disease. Several publications have
suggested that behavior, especially hind limb scratching, is an important driver of UD.
Therefore, it was hypothesized that B6 mice would demonstrate a tendency to scratch more
either spontaneously or when provoked by epidermal barrier disruption or the spray test. In
contrast with this hypothesis, there was no evidence that B6 scratched more frequently or for
longer durations than the other commonly used strains tested. In fact, under some conditions the
B6 mice scratched significantly less than the other mouse strains. These findings do not support
the idea that a strain-related tendency toward exaggerated scratching behavior under resting or
epidermal barrier disruption conditions predisposes B6 mice to UD.

In previous studies B6 mice exhibited robust scratching behavior compared to other
strains with low doses of intradermal pruritogens (Green et al. 2006, Inagaki et al. 2001). The B6
mice were relatively susceptible to low doses of the exogenous pruritogen, however their
maximum time spent scratching was less than other strains at the peak of their dose response
curve. The present study did not assess degrees of epidermal barrier insult but the low maximum
scratching frequency and duration is similar to the observations by Green et al. Also, although
increased pruritus as assessed by the spray test was previously associated with later UD
development, the findings of our study did not find a significant increase in scratching in the UD
predisposed B6 and the other strains tested. The study by Dufour et al. only used B6 mice so it is
possible that the spray test could have predictive value for individual animals within the strain,
but is not associated with the B6 strain's predisposition to UD development. None of the B6

mice in this study developed UD during the acclimation period or the interval between baseline and post-epidermal barrier injury behavior testing so an association with spray test scratching behavior could not be assessed.

In this study the ICR mice, but not the inbred strains, showed a highly significant difference between morning and evening scratching frequency. A similar tendency was seen in the B6 and DBA/2 mice although the difference was not statistically significant. Grooming has been shown to be a self-calming and displacement behavior for rodents in novel environments (Spruijt et al. 1992, Tuli et al. 1995), and scratching was often observed to occur at the beginning or end of grooming sessions. The baseline and post-tape stripping tests occurred several weeks apart, and it is possible that the differences in scratching behavior between the first and second filming for baseline and post-tape stripping may be related to familiarity with the testing set up (and less anxiety related grooming behavior) rather than the time of day the filming occurred. Alternatively, this mouse stock may potentially scratch more in the evening than in the morning. Although some studies have controlled for time of day by performing behavior testing during a specified window of time each day (Dufour et al. 2010, Ohmura et al. 2004), no studies could be found that compared morning and evening scratching behavior in mice.

There are several possible interpretations for the relatively low scratching frequency and duration seen in the B6 mice in this study. It is possible that scratching behavior is not an initiating factor but rather a secondary consequence of UD. In one study where scratching behavior was assessed every two weeks until the animals were harvested for tissue collection, mice had a marked increase in scratching frequency when UD was present but there was no increase at the time point 2 weeks preceding the diagnosis of UD (Hampton et al. 2012).

Alternatively, a dysfunction of the nerves innervating the epidermis could lead to a combination a paresthesia and loss of protective pain during scratching events. In human medicine there is a recognized condition called trigeminal tropic syndrome that combines intractable neuropathic itch and the profound loss of cutaneous sensation leading to painless self-injurious scratching. Similarly aged B6 mice with a cutaneous neuropathy could have hypoesthesia which manifests as a relatively infrequent scratching but, once provoked to scratch by secondary bacterial infection or other compounding factors, they may lack the necessary feedback to stop scratching before they harm themselves. Intraepidermal nerved fiber density (IENFD) has been used to assess for neuropathy secondary to diabetes and experimental models of neuropathy in rodents and could be used in the study of UD (Brewer et al. 2008, Horiuchi et al. 2005, Kou et al. 2012). IENFD assessment is currently being performed on frozen tissue sections collected from the various strains during this experiment to evaluate the possibility of abnormal cutaneous innervation in B6 mice.

One important limitation is that this study only addressed scratching behavior following acute injury. Differences in scratching behavior due to aberrant feedback mechanisms or wound repair that occur more than 24 hours after epidermal barrier disruption would not be apparent. In addition, intensity of scratching could be strain related and of significance to UD development, but was not assessed in this study. Both of these areas are promising avenues for further study.

The inbred strains used in this study were chosen because they are commonly used and are classic comparison strains for the B6. The BALB/c has Th2-biased immune responses, which has been contrasted with the Th-1 biased B6, and is common in infectious disease and allergy studies (Flurkey et al. 2009, Percy and Barthold 2007). The DBA/2 is a historic comparison

strain that is frequently crossed with the B6 for F1 hybrids and a large number of DBA/2xB6 recombinant inbred strains exist (Flurkey et al. 2009). There is the potential to use these recombinant inbred strains to interrogate genetic influences in UD. While these provide reasonable comparison strains for the B6 it should be noted that they should not be considered representative of inbred strains as a whole. Therefore, the findings from this study should be generalized with caution.

In this report we demonstrated a relatively low frequency and duration of scratching behavior in UD prone B6 mice as compared to BALB/c, DBA/2, and ICR mice. This is contrary to what would be expected if excessive scratching during typical grooming behavior or following mild skin insults were the underlying cause of UD in this strain. Understanding the etiopathogensis of this condition will ultimately be important for improving the clinical management of UD and further study of potential behavioral, metabolic, and neuropathic mechanisms is critical.

Table 3.1. Mean scratching frequency by strain and time point. Scratching behavior was recorded at baseline time points, 5 h post-tape stripping, and 17 h post-tape stripping. Behavior recordings were performed starting at 17:15 (PM) and the next morning at 07:00 (AM) under both conditions. In addition to the mean, the standard deviation (SD) and the 95% confidence interval (95% CI) are displayed in the table below.

| Observation	Mean	SD	95 % CI
C57BL/6			
Baseline PM	6.7	4.8	+/- 2.7
Baseline AM	4.1	2.6	+/- 1.5
5 hr Post (PM)	22.3	10.8	+/- 6.1
17 hr Post (AM)	14.3	9.4	+/- 5.3
BALB/c			
Baseline PM	28.7	18.4	+/- 11.4
Baseline AM	19.9	17.8	+/- 11.0
5 hr Post (PM)	24.8	17.9	+/- 11.1
17 hr Post (AM)	24.3	8.4	+/- 5.2
DBA/2			
Baseline PM	35.9	22.0	+/- 15.2
Baseline AM	23.0	11.7	+/- 8.1
5 hr Post (PM)	45.8	18.2	+/- 14.6
17 hr Post (AM)	33.0	9.7	+/- 7.8
ICR			
Baseline PM	17.5	7.0	+/- 4.3
Baseline AM	6.8	4.6	+/- 2.9
5 hr Post (PM)	41.8	10.3	+/- 6.4
17 hr Post (AM)	16.7	10.5	+/- 6.5
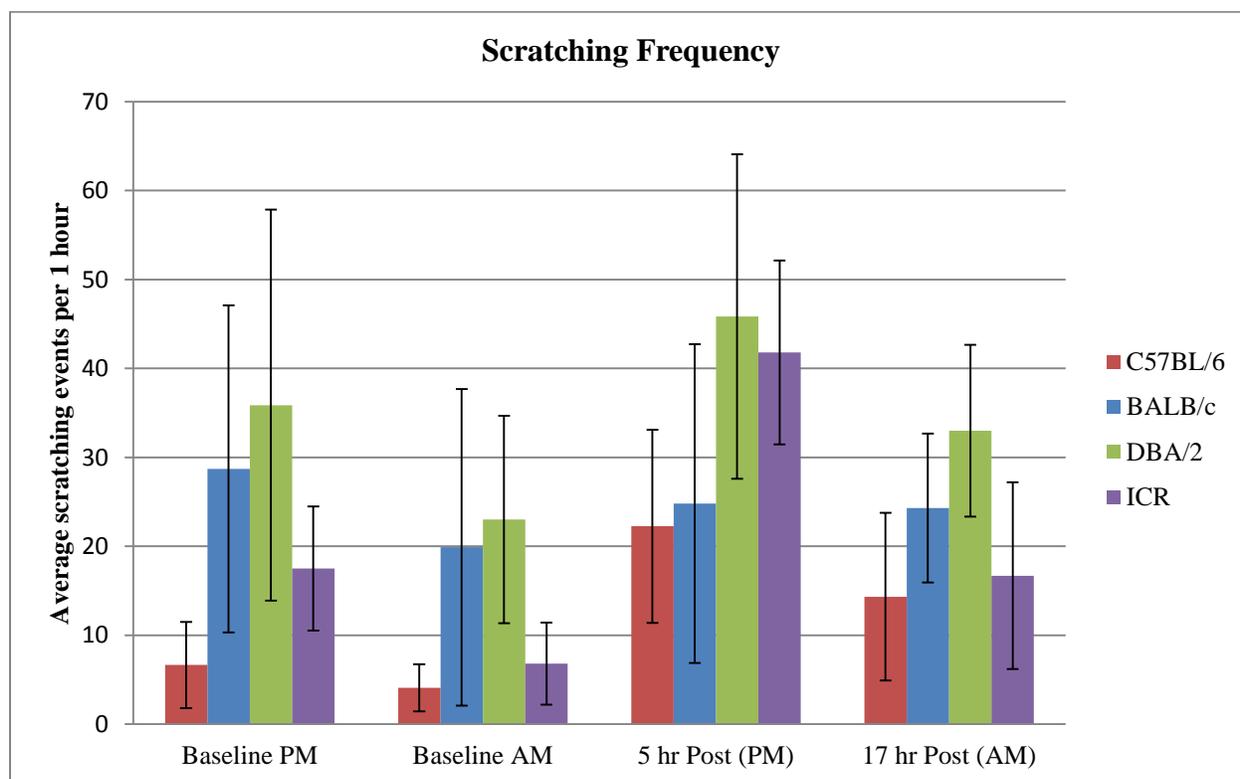
Figure 3.1. Scratching frequency by strain and time point. Scratching behavior was recorded at baseline time points, 5 h post-tape stripping, and 17 h post-tape stripping. Behavior recordings were performed starting at 17:15 (PM) and the next morning at 07:00 (AM) under both conditions. Scratching frequency is displayed as mean scratching frequency by strain +/- standard deviation depicted by the error bars. Strains included the C57BL/6 (n=12), BALB/c (n=10), DBA/2 (n=6-8), and ICR (n=10). At baseline, B6 mice scratched significantly less than the BALB/c (p= 0.0042 PM), DBA/2 (p= 0.0068), and ICR (p= 0.00080) mice at the PM time point. At the Baseline AM time point the B6 scratched significantly less than the DBA/2 (p= 0.007). At 5 and 17 hours post injury the B6 mice scratched the least. This lower frequency of scratching events was statistically significant compared to the ICR mice (p= 0.0004) at the 5 h post injury time point. At 17 h post injury the B6 scratched significantly less than the DBA/2 (p= 0.0009).
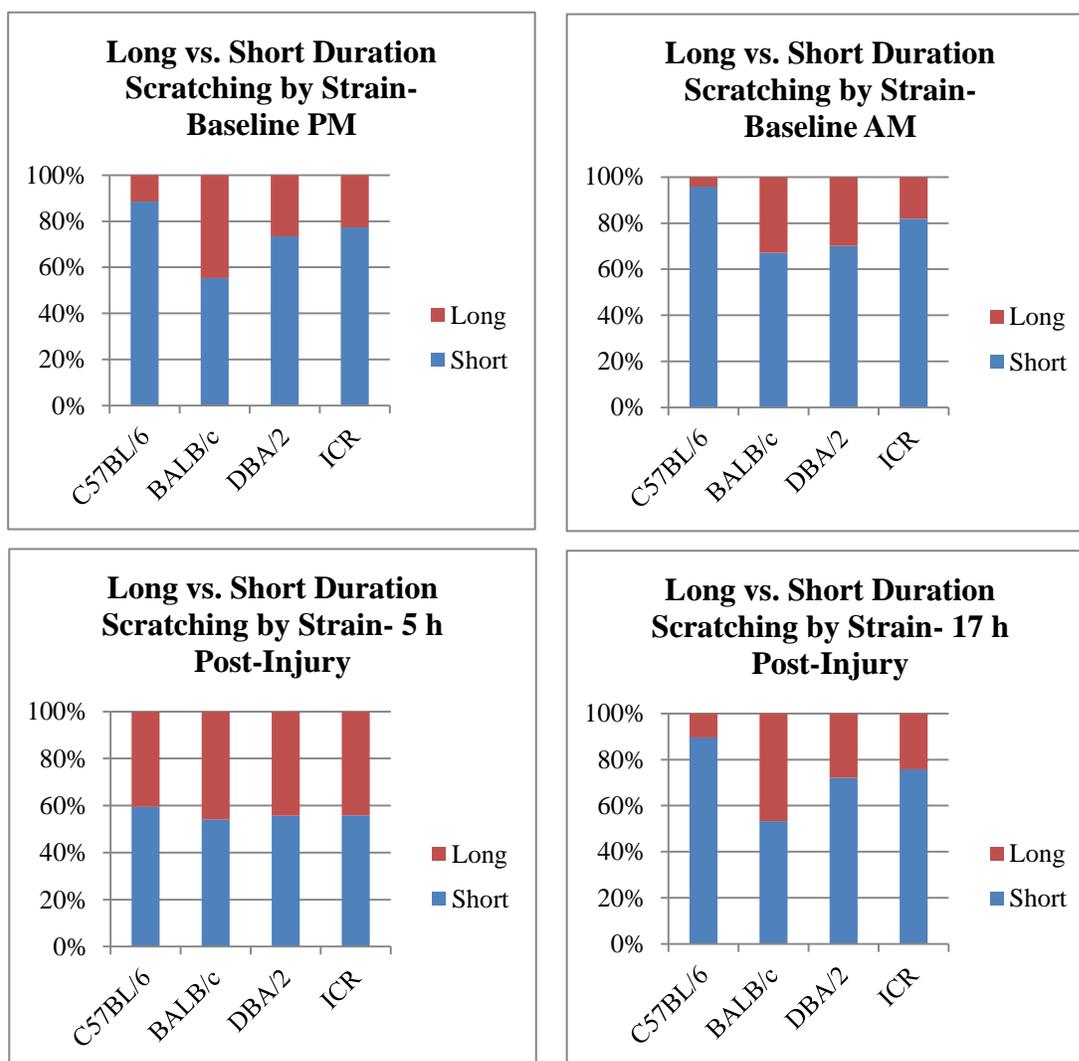
Figure 3.2. Percentage of short and long duration scratching events by strain. Short scratching events were defined as <3 seconds in duration and long scratching events were defined as those lasting ≥3 seconds. Scratching behavior was recorded at the baseline time points, 5 h post-injury, and 17 h post-injury. Strains included the C57BL/6 (n=12), BALB/c (n=10), DBA/2 (n=6-8), and ICR (n=10). The B6 mice had the highest percentage of short duration scratching events at baseline and 17 h post-injury.
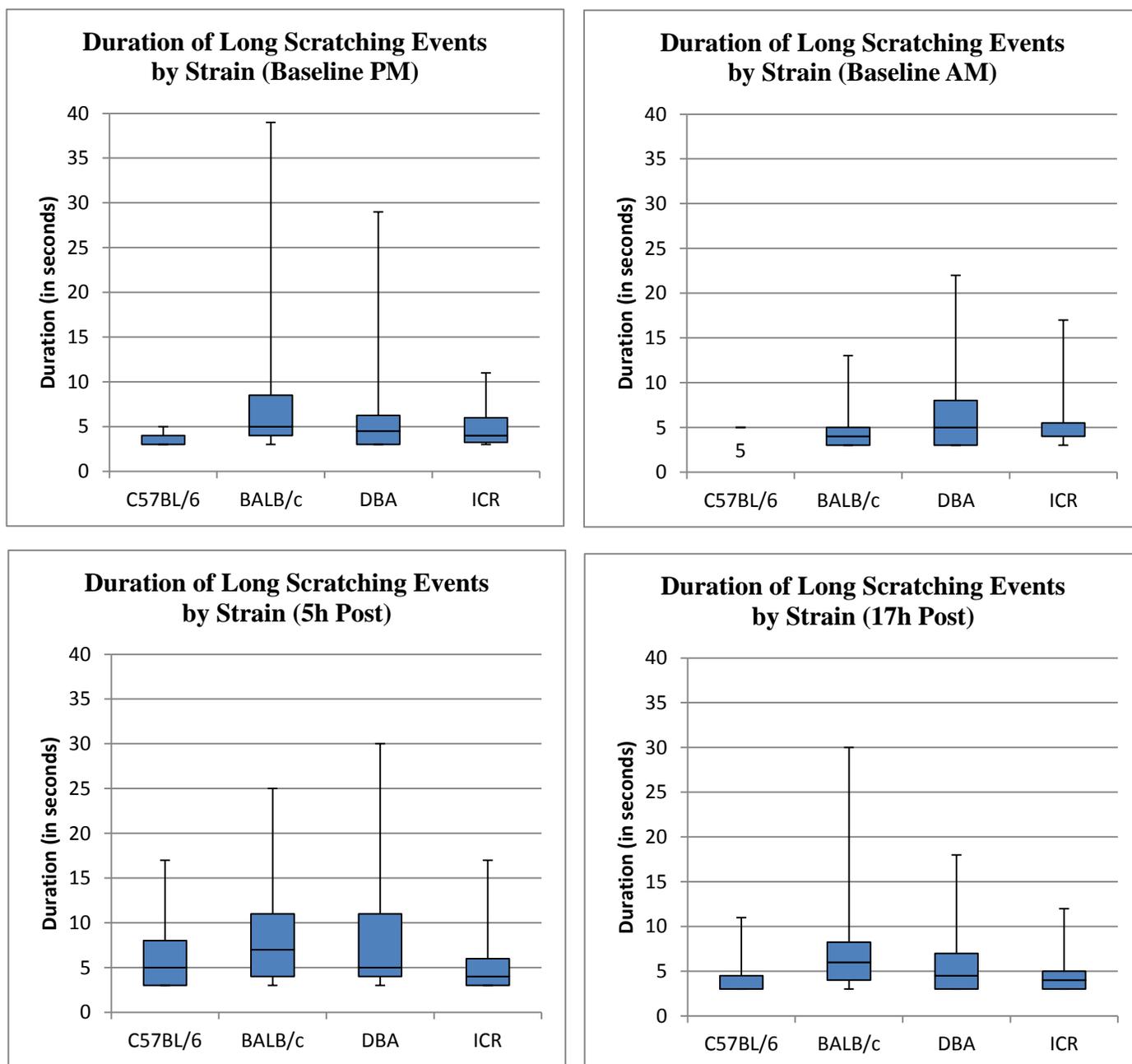
Figure 3.3. Median duration of long scratching events by strain and time point. Scratching behavior was recorded at baseline time points, 5 h post-tape stripping, and 17 h post-tape stripping. Strains included the C57BL/6 (n=12), BALB/c (n=10), DBA/2 (n=6-8), and ICR (n=10). Median duration was very similar between strains with no statistically significant difference between the B6 mice and other strains. At the Baseline AM time point only one scratching event for the B6 mice had a duration of >3 seconds, so the value is depicted instead of the box and whisker plot.
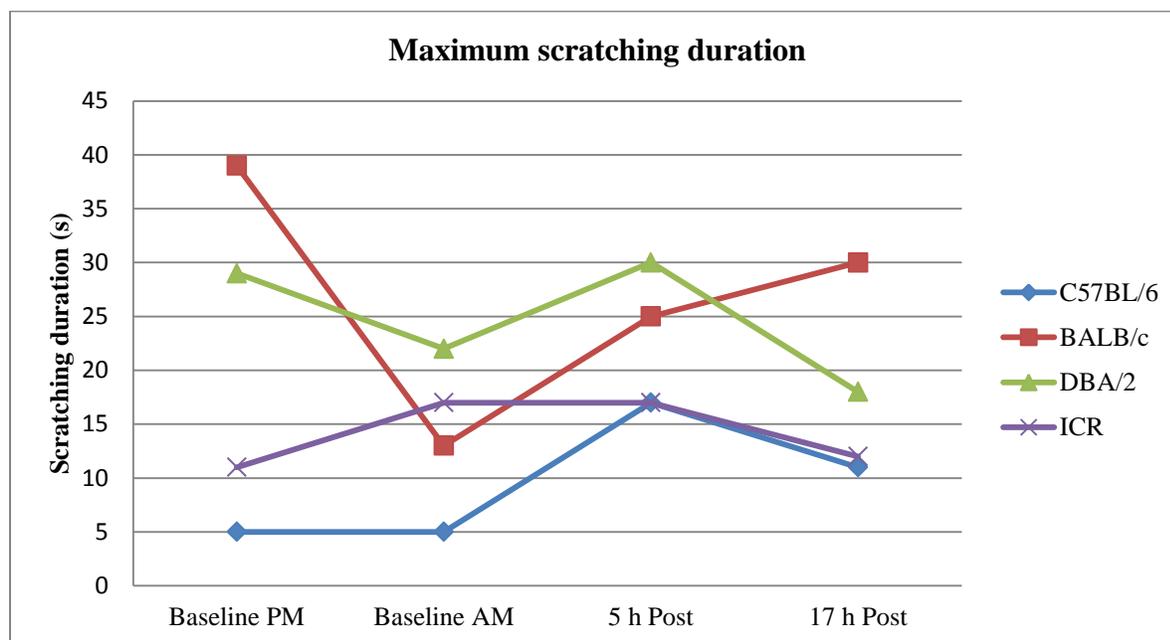
Figure 3.4. Maximum scratching duration. The single longest scratching event recorded at each time point by strain is illustrated above. Scratching behavior was recorded at baseline time points, 5 h post-tape stripping, and 17 h post-tape stripping. Strains included the C57BL/6 (n=12), BALB/c (n=10), DBA/2 (n=6-8), and ICR (n=10). B6 mice did not have the highest maximum scratching event at any time point.
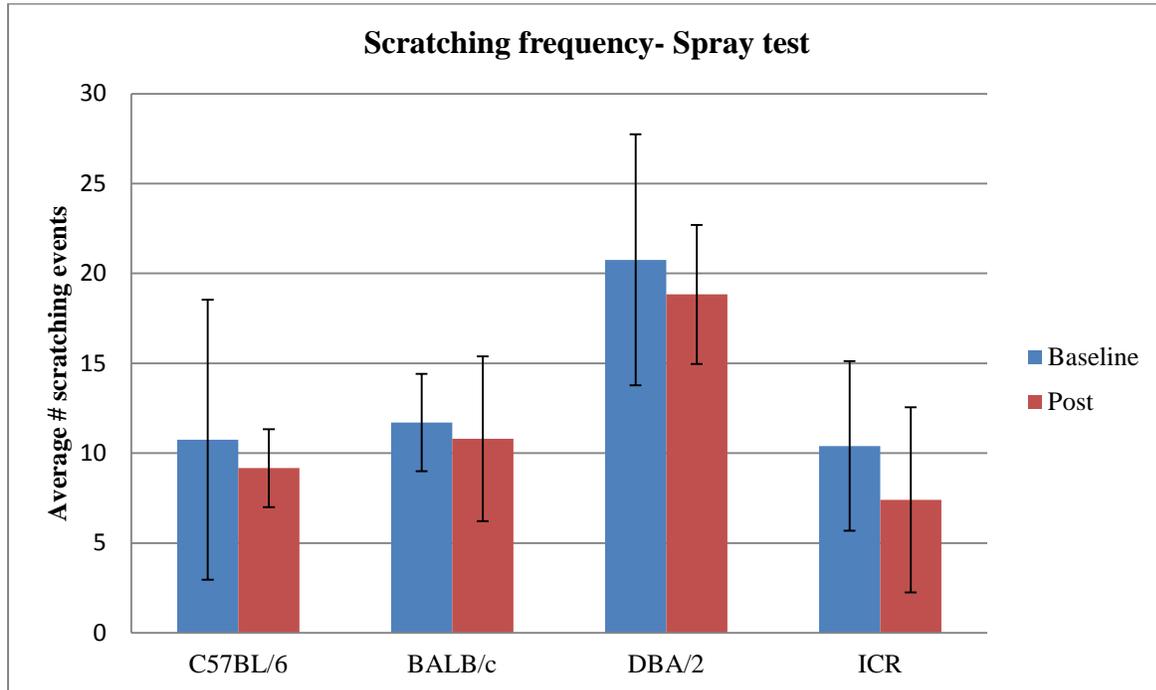
Figure 3.5. Scratching frequency during the 15 min spray test. Scratching behavior was recorded at the baseline time points, 5 h post-injury, and 17 h post-injury. Strains included the C57BL/6 (n=12), BALB/c (n=10), DBA/2 (n=6-8), and ICR (n=10). Scratching frequency is displayed as mean scratching frequency by strain +/- SD depicted by the error bars. The B6 mice scratched significantly less than the DBA/2 at baseline (p= 0.008) and 17 h post (p= 0.0009). In addition, the post injury scratching frequency was significantly different from baseline in the ICR (p= 0.0429). There was no significant difference in scratching frequency pre- and post-epidermal barrier injury in the B6, BALB/c, and DBA/2 (p >0.05).
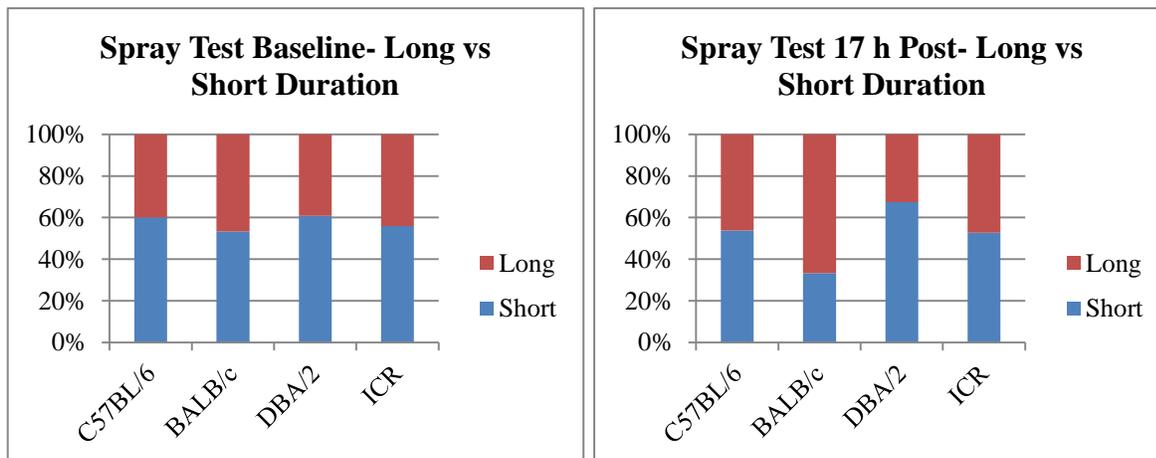
Figure 3.6. Spray test duration as a percentage of short vs. long scratching events by strain. Short scratching events were defined as <3 seconds in duration and long scratching events were defined as those lasting ≥3 seconds. Scratching behavior was recorded at the baseline time points, 5 h post-injury, and 17 h post-injury. Strains included the C57BL/6 (n=12), BALB/c (n=10), DBA/2 (n=6-8), and ICR (n=10).
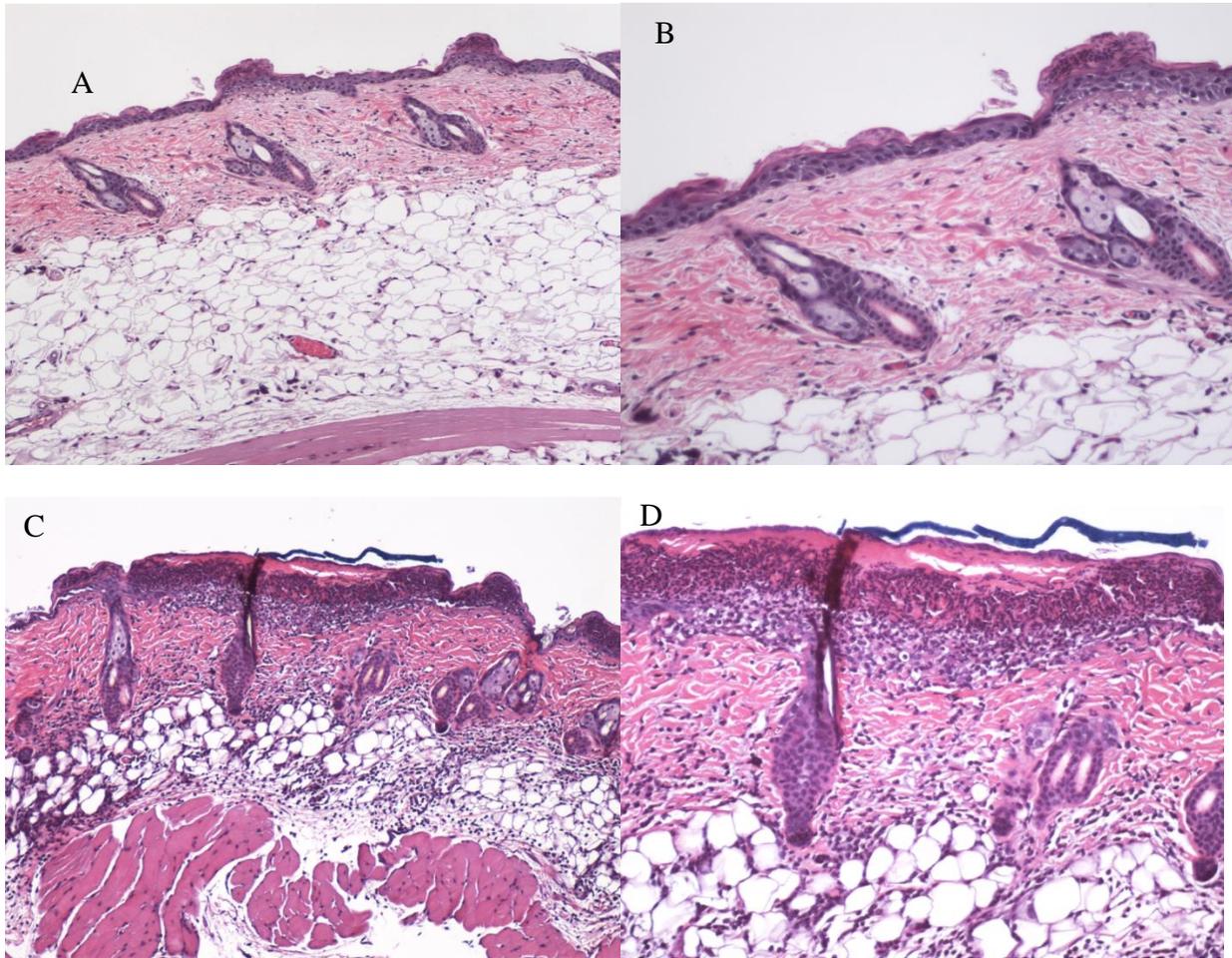
Figure 3.7. Histology of tape stripped skin. Representative histology images of the suppurative dermatitis with pustules seen during evaluation of the tissues. A: H&E staining, 100x magnification. Small discrete pustules seen on the epidermal surface. B: H&E staining, 200x magnification. A greater magnification of the lesions in the preceding image (A). C: H&E staining, 100x magnification. Larger coalescing pustules with mixed inflammatory infiltrate seen in the dermis and adipose tissue. D: H&E staining, 200x magnification. A greater magnification of the lesions in the preceding image (C).

Chapter 4: Conclusion

The B6 mouse is arguably the work horse of biomedical research. It was the first non-human animal to have its genome sequenced (Flurkey et al. 2009). A search for "C57BL/6" in PubMed (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, Maryland) produced over 32,000 articles. Also, a wide range of phenotypic and genetic information is well characterized for the strain providing a range of tools for research that is unprecedented for veterinary species (Bogue and Grubb 2004, Bryant 2011, Grubb et al. 2004). As such it is truly unfortunate that UD, a condition that affects many mice of the strain, is so poorly understood.

In performing a standard literature search it was apparent that there is an accepted typical clinical appearance and progression for UD although there is significant disagreement regarding other aspects of the condition. Generally it was described as occurring in aged C57BL/6 mice with affected animals described as having a rapid onset of intense scratching and erosive or ulcerative lesions that were refractory to a range of treatments. Inconsistently there were reports of an increased incidence of the disease in female mice, mice exposed to certain diets, advanced age, and several seasons. These inconsistencies indicated a need for a systematic review to better assess the evidence for commonly cited UD risk factors and treatments.

The systematic review highlighted some priorities for further study and revealed the generally low quality of research on UD, especially regarding treatments. Of the four risk factors evaluated, diet was the most consistently associated with altering UD risk. Particularly *ad libitum* feeding as opposed to a calorically restricted diet was associated with an increased risk of UD in the studies where this was evaluated. Dietary modulation of UD is, therefore, a very promising

avenue for further study. In addition, an increased incidence in female mice was noted in studies evaluating dietary interventions suggesting that there may be an important interaction between diet and sex in the pathogenesis of the disease. Regarding the quality of the available literature, the reports discussing risk factors for UD had highly variable or poorly described inclusion or diagnostic criteria. Also, there were very few controlled studies of UD treatments. Future research should aim to address these shortcomings in the current literature.

The standard literature review also revealed that, of the many proposed etiologies, there was increasing evidence for a central role of scratching behavior in UD progression. This inspired the hypothesis that UD may be the result of excessive scratching behavior by the B6 strain under normal conditions or when provoked by epidermal barrier injury. It was reasoned that if B6 mice scratched exuberantly as part of their normal grooming repertoire that UD may be an extension of this strain-related trait. Surprisingly, B6 mice scratched less frequently than the other mice tested when observing both spontaneous and provoked scratching behavior. Scratching duration was then examined, as fewer but longer scratching events could be equally or more damaging than a higher number of shorter scratching bouts. In assessing scratching duration, it was found that the B6 mice didn't have a higher number of long scratching bouts and that the maximum scratching duration was not longer than the other mice. The results of this study fail to support the initial hypothesis. However, this "negative result" has important implications for further studies of UD pathogenesis. The relatively low rates of scratching observed are consistent with other reports where the onset of scratching is associated with the onset of UD, but not necessarily preceding it. Further characterization of the initiation of pruritus as compared with the appearance of skin lesions will further elucidate the role scratching plays in UD progression.

Although the cause of UD is still unknown, I suspect that the etiology of UD is ultimately multifactorial with significant genetic, metabolic, and microbial components. A simply inherited genetic defect is unlikely given that all B6 mice have essentially the same genotype but they do not all share the same phenotype in terms of whether or not they get UD. Instead of B6 mice having a "UD gene" is it more likely they have an inherited epidermal barrier, immunological, or other trait that increases their risk for the condition but isn't sufficient to cause UD on its own. Similarly it could be the unique constellation of traits fixed in the B6 strain that puts them at increased risk. Nonetheless, the predilection for UD in B6, C57BL/10, and closely related mutant strains suggests that a heritable component is important for UD risk. Nutrition and metabolic processes are likely involved as well given the effects of diet reported and the absence of UD in insulin receptor substrate 1 knock out mice. Presumably this would be an indirect effect of nutrient sensing and signaling on the immune system or nervous system that results in chronic itch or susceptibility to opportunistic infection by epidermal microbes. Secondary infection of diseased skin, for example with atopy, can exacerbate pruritus. While antibiotic therapy is not curative in mice with UD there is likely an important role for secondary bacteria in amplifying the itch-scratch cycle, thus exacerbating the self-trauma that is inflicted.

One could also speculate that the unnatural conditions of the laboratory have a role as well. Inbreeding, which is common practice for maintaining laboratory mice but generally avoided in other husbandry systems, is historically associated with genetic disease. During informal conversations about my research with veterinarians outside of the laboratory animal field and laypersons concerns have been raised about inbreeding as a cause for UD. Also, in the laboratory mice are housed in cages that are much smaller than the typical foraging ranges for wild rodents and represent a relatively barren environment compared to the complexity of wild

habitat. Although it is standard practice to socially house mice and provide enrichment unless there is a scientific or medical justification for not doing so, the housing conditions of domestic laboratory mice have been speculated to put mice at risk for abnormal behavior (Dufour et al. 2010). While laboratory factors such as these may contribute to UD risk they are unlikely to play a major role. While B6 mice have been maintained as an inbred strain for many generations there are other mouse strains which do not get UD, such as the DBA/2, that have been intensely inbred for the same number of generations or even longer. Therefore, UD does not appear to be simply a consequence of being inbred. Similarly if the cage environment alone was responsible for inducing UD then it would be seen more commonly in other inbred strains or outbred mice which are housed in the same manner. Given the narrow range of the related inbred strains and genetically engineered mice on those backgrounds that get UD, laboratory conditions do not appear to be sufficient to cause UD but may have a minor role.

There is a lot left to be learned about UD, but some especially promising future directions are suggested by this work. The association between *ad libitum* diets and UD could be explored using methods already widely employed. For example, a prospective study evaluating UD onset in association with blood glucose concentrations, body weight, and metabolic assessments may be enlightening. Likewise it would be interesting to compare scratching behavior between B6 mice on an *ad libitum* diet with calorically restricted controls. Another future direction which is currently being undertaken is to assess the cutaneous innervation of B6 mice as compared to several other genotypes of mice that do not get UD. Based on the relatively low frequency and duration of scratching demonstrated in this report, the inconsistent evidence of a primary dermatologic lesion, and the high risk of self-injurious scratching seen with pruritic neuropathies in human medicine a potential neuropathic mechanism for UD should be considered.

Immunohistochemical staining for intraepidermal nerve fiber density assessment is one way to assess this possibility. Additionally, sensory testing or receptor staining of neurons in the dorsal root ganglia could be employed. Although progress has been slow in determining the etiology of the UD, the studies reported here provide some additional steps forward in addressing this critical need.

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Appendix A: History, basic nomenclature, and substrains of C57BL/6

This is a very brief history and nomenclature overview meant to clarify basic concepts to provide background for this thesis. For more information on these topics several excellent resources are available (Bryant 2011, Flurkey et al. 2009).

Inbred mice are produced by 40 or more generations of brother-sister mating. By the 40th generation the resulting mice are considered almost genetically identical. This provides the opportunity to minimize experimental variability due to genetic differences in animal studies. Inbred mice, such as the C57BL/6, are referred to as strains in contrast to outbred mice, which are referred to as stocks.

The B6 mouse was developed in the 1920s by Dr. Clarence Cook Little from female 57 and male 52 from Miss Lathrop's stock. This cross also gave rise to the C57BL/10, C57L, and C57BR strains.

Substrains may be developed if a group of mice of a given strain are maintained in an isolated colony for 20 or more generations due to minor genetic variations, genetic drift, and spontaneous mutations. Substrains are designated by "/" followed by the laboratory code. Several common substrains of the B6 mouse are maintained at laboratory rodent vendors and were used in the reports referenced in this thesis:

C57BL/6J- Jackson Laboratory substrain

C57BL/6Crl-Charles River Laboratories substrain

C57BL/6NNia- Originally from the NIH then transferred to the National Institute for Aging

C57BL/6OlaHsd- Harlan Laboratories substrain

Appendix B: Problems and solutions

Mice

"Retired" breeders

Retired breeders were used as source of aged animals for the experimental work described in Chapter 3. This was done as a means of reduction, as these mice otherwise would be culled, and as a means of reducing study costs. Unfortunately, there were several challenges I faced using these study animals.

Of the 45 mice used in the study 12 were pregnant upon arrival. This was a source of unintended variation as the pregnancy status, litter size, and delivery date could not be controlled for. There were also differences by strain with 7 out of 10 DBA mice and 4 out of 15 B6 mice delivering pups, while none of the BALB/c or ICR mice had litters. Given that genotype was the variable of interest this disproportionate pregnancy rate was problematic. In addition, the large number of animals made exclusion of pregnant mice from the study impractical. To address this issue a long acclimation period (6 weeks) was instituted to accommodate gestation, pup rearing, and recovery from lactation.

Another issue was the poor body condition of many of the DBA/2 and BALB/c mice. Although this may have been related to gestation and lactation for the DBA/2 mice, none of the BALB/c mice had pups while they were in the facility. The solution for this was to provide all mice with a highly palatable gel diet for the first week after arrival and at cage change (every two weeks) thereafter. Diet cups last for several days and provided an intermittent nutritional boost. The gel diet was provided to all the mice so that dietary exposures would be similar amongst all

the subjects even though dietary supplementation was not needed for the ICR mice and many of the B6 mice.

Unexplained DBA/2 deaths

Two DBA/2 mice died during the initial acclimation period and two more died in the three week interval between their initial filming and the epidermal barrier disruption procedure. None of the mice died after experimental procedures such as behavioral observation or anesthesia. In all cases the mice had no premonitory signs and were found dead during routine morning health checks by husbandry staff. Although many of the DBA/2 mice were thin (BCS 2.0-2.5/5.0) most of the BALB/c mice were in a similar body condition. Parity may have been a factor as 7 out of 10 DBA/2 mice, unlike the similarly thin BALB/c mice, gave birth to a litter shortly after arrival. Of the 4 DBA/2 mice that died ¾ had delivered pups during the initial acclimation period. Of the 3 that gave birth all had small litters of one to three pups. DBA/2 #3 died two days after her litter of 2 pups was weaned. DBA/2 # 8 died one week after her 2 pups were weaned. For these two individuals the demands of pup rearing may have contributed to their deaths, although the of the other two DBA/2 mice that died one died 2 months after weaning her litter and one had not delivered pups during her time at OSU. Diagnostic necropsy of the dead mice was unrewarding due to marked autolysis and partial cannibalism in all cases. Gross necropsy of the other DBA/2 mice in the study typically revealed poor body condition (which had been noted antemortem) and cardiac calcinosis. Epicardial and myocardial calcification is considered a common finding in the strain, especially in female mice (Percy and Barthold 2007). It is possible that cardiac dysfunction, such as an arrhythmia, secondary to

myocardial calcification may have been the cause of death for the 4 female DBA/2 mice, although a definitive cause of death cannot be determined.

The death of the DBA/2 mice meant that there were cages with 3 mice per cage instead of 4. To address this difference in cage density the replacement B6 mice were used to keep the cage density equal amongst all 10 cages of experimental subjects. As two of the DBA/2 mice died before baseline filming, the 2 B6 mice that replaced them underwent behavior observations and tape stripping. In the case of the two B6 mice that replaced the DBA/2 mice that died after baseline filming, they were not tape stripped or filmed for scratching behavior. They were simply used to maintain housing density. Since mixing of social groups could have unintended behavioral consequences any cage that had a replacement mouse added were allowed to acclimatize for 1 additional week before behavioral observations.

Estrous cycles

Female mice were used in the work described in Chapter 3 because it allowed for aged mice to be reassembled into new social housing units that included one mouse of each genotype per cage. Male mice, particularly older male mice, are likely to fight which presents health and welfare concerns regarding cohousing. Several additional considerations with female mice, however, should be addressed. First, the stage of the estrous cycle of the mice could have affected their behavior. Vaginal cytology or histological analysis of the reproductive tract were not performed as part of this study and could be used as a potential refinement for future work. Several pheromone effects are well documented in mice. One such effect is the Lee-Boot effect which results in the suppression or synchronization of estrous cycles in female mice that are group housed in the absence of males. The mice used in this study were group housed and there

were no male mice housed in that animal room. Therefore, it was likely that the mice in this study were not cycling or had synchronized with the other mice in their cage. However, as stated above stage of estrous cycle was not assessed.

Parity and UD

It is unknown what the effect of parity is on UD. Several studies have noted an increased risk in female mice, but none of these studies described whether the mice had been used for breeding and, if so, the number of litters that had been raised by the mouse. These studies were mostly diet trials that likely used aged subjects that had not been previously used as brood stock. The study described in this thesis work used retired breeders that likely had one or more litters prior to being acquired for this study. The hormonal effects of these reproductive events may have an effect on UD, but no research has been performed that addresses parity and UD.

Conclusions

Although the solutions used allowed for the continuation of study, there was unintended variation unrelated to the experimental protocol that was introduced by the use of these retired breeding females. If I were to repeat this study I would use purpose bred aged mice as opposed to retired breeders to minimize the problems seen with inconsistent condition and pregnancy status among the experimental subjects. In studies such as these some age related morbidity and mortality cannot be entirely avoided. However, the long acclimation and the effect of pregnancy and lactation on mouse health were undesirable complications that could have been eliminated by the use of mice that had been intended for use as aged mouse models.

Technical difficulties

<u>Observation chambers</u>

The observation chambers used to record scratching behavior 26.7 cm x 36.8 cm x 21.6 cm deep. Although I had hoped that the depth of the chambers would be sufficient to prevent mice from jumping out of the chambers during filming there were many occasions where the mice were able to jump up onto the wall of the chamber. Fortunately none of the mice tried to venture beyond the wall of the chamber and attempt to escape. However, when this happened I needed to interrupt filming briefly to put the mice back on the floor of the chamber. Doing so did not appear to disrupt the mice greatly, although, presumably there was an effect on their behavior. I originally did not want to use any type of top or lid to the cage as I was concerned this would interfere with seeing scratching events. After one mouse decided to try jumping out 10 times, I had a Plexiglas top made. This stopped the mice with an inclination to jump from going anywhere and was transparent enough that there was no interference with observing their behaviors. For similar studies in the future I would use a Plexiglas top like this for all of the chambers from the initial baseline filming onward.

<u>Hair removal</u>

In order to tape strip the skin for the induction of epidermal barrier disruption I first had to remove the hair from the area. At the outset I planned to simply remove the hair with clippers. Unfortunately this left a layer of short hair and more aggressive clipping to reduce the hair left behind put the mice at risk of nicks from the clipper blade. The solution was to do an initial clipping to shorten the hair and use a depilatory cream to remove the remaining hair without the risk of "clipper burn." This did an acceptable job of removing the hair although the end result

could be slightly patchy and there was some potential for the depilatory cream to cause some irritation or mild inflammation independent of the tape stripping procedure. Another technique that has been used in the literature is using a cold waxing procedure to remove the hair. If I were to repeat this experiment I would be inclined to try this method as it may have resulted in more uniform hair removal.

<u>Histology</u>

Based on previous studies that described that mice are unable to reach the caudal dorsum area to scratch I wanted to analyze the cranial and caudal dorsal aspects of the tape stripped and control skin separately. The end result was subdividing the tissue collected into tape stripped and scratching accessible, tape stripped but inaccessible, control side scratching accessible, and control inaccessible. Initially I tried using a divided cassette but the skin segments would not remain in a good orientation for sectioning. This problem was addressed by taking the tissue destined for fixation as a strip, marking the cranial aspect with tissue dye, and rolling the strip of tissue onto the wooden part of a cotton-tipped applicator. This was performed for both the tape stripped and control side resulting in two tissue "rolls." The tissue was then fixed overnight, placed in a tissue cassette, and submitted to the Veterinary Diagnostic Laboratory. This technique worked rather well. The only minor issue was that some of the tissues would unroll slightly resulting in a partially oblique section. This generally comprised only a small area of the tissue on the slide. Overall I would recommend this approach for histologic sampling of mouse skin, although it could potentially be more challenging to make tissue "rolls" if the hair was not removed.

Tape stripping quality control

In evaluating the histology there was a consistent pattern of pustular suppurative dermatitis in response to tape stripping. The dermatitis ranged in severity from mild lesions with superficial keratinocytes removed but very rare inflammatory cells to severe lesions with large portions of the tissue section having coalescing pustules, deeper inflammatory cells, and edema. One potential refinement for future studies would be to have a means of assessing consistency in the degree of epidermal barrier disruption created. For example, one could use transepidermal water loss (TEWL) to assess the degree of barrier disruption. This method has been previously described and in some cases was the end point for tape stripping rather than a specific number of tape application/removal cycles. Similarly it was difficult to assess which changes were due to the tape stripping procedure and which changes were due to the scratching and grooming behavior that occurred afterwards. One way to evaluate this would have been to have a subset of animals that were euthanized following tape stripping but prior to anesthetic recovery. Likewise bandaging and E-collars could have been used to allow the epidermal barrier insult to age 24 hours in an awake mouse while preventing secondary injury. This has been reported to be technically challenging as mice that are motivated to scratch can be very good at removing these types of devices (Akiyama et al. 2012).

Scratching behavior recording

There were several challenges to analyzing scratching behavior that arose over the course of the study. The overhead filming angle provided a good means of capturing the behavior of several mice at once, but at times it could be difficult to tell certain grooming behaviors from hind limb scratching. For instance, hind paw licking/grooming looked very similar on film.

Similarly if mice were at a slightly oblique angle and were scratching the ventrolateral aspect of the trunk this could be hard to appreciate. To address this issue I only recorded scratching events that I was confident were scratching events, while behavior that could have been scratching but was hard to interpret was not counted. This was done by watching ambiguous behaviors three times. If at the end of the repeated viewing I was still not confident the mouse was scratching the event was not recorded. Another solution would be the use of automated scratching counters, such as the MicroAct system which has been previously described (Akiyama et al. 2010, Akiyama et al. 2012). The use of an automated system would also have addressed the second challenge which was the time intensive nature of analyzing the filmed behaviors. Given the number of cages and the number of time points I calculated that it took me 90 hours to watch the video once. This, of course, does not include the time needed once I accounted for rewinding and re-watching segments to confirm that mice were indeed scratching or to document the length of time for long scratching events.

In previous studies scratching has been quantified in terms of number of scratching events or by total time spent scratching. In the literature studies rarely quantified both frequency and duration in the same report. Based on the behavior seen during a pilot study a cut-off for short and long duration scratching was determined. While this was a helpful addition to the scratching frequency measure, in hindsight recording duration in seconds for all of the scratching events would have been helpful. By doing so scratching duration could have been analyzed as a continuous variable rather than a categorical one. By doing so it would have created an opportunity to perform different and more conceptually straight forward statistical analyses.

Conclusion

Valuable data were obtained during this research, however all projects have unanticipated challenges. The issues discussed above may be useful tips and considerations for future work using similar techniques. By building off of the lessons learned from prior studies hopefully future investigators will be able to achieve success in comparable studies without having to "reinvent the wheel."