SCIENTIFIC SESSION PRESENTATIONS

HUMAN ATOPIC DERMATITIS: FROM LABORATORY RESEARCH TO BEDSIDE

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INTRODUCTION

Human atopic dermatitis (AD), similar to canine AD, are the most common inflammatory skin diseases found in their respective species. Each of these disease homologues are associated with marked pruritus, xerosis and a skin rash associated with chronic inflammation with intermittent acute flares of skin disease. AD often starts during infancy and early childhood but can persist into adulthood. Unlike canine AD, human AD is often associated with the atopic march which refers to the process of developing asthma after having childhood AD. In human AD, wide variations in prevalence have been observed within countries inhabited by similar ethnic groups, suggesting that environmental and immunologic factors play a critical role in determining the clinical phenotype of this genetically transmitted disease. This lecture will review recent advances in our understanding of the pathobiology of human AD and disease management approaches for control of the skin barrier dysfunction and inflammatory responses associated with AD.

EPIDERMAL BARRIER DYSFUNCTION

Patients with AD have widespread xerosis of the skin even involving non-lesional skin. Functional epidermal barrier dysfunction has been documented by demonstration of increased transepidermal water loss. This impairment of the skin barrier function in AD leads to increased allergen absorption and lowered irritancy threshold contributing to the cutaneous hyperreactivity characteristic of AD. The mechanism for epidermal barrier dysfunction in AD is highly complex with multiple abnormalities identified. These include reduced lipid (e.g. ceramides) content, increased protease activity, low protease inhibitor levels and loss of key epidermal barrier proteins, such as filaggrin. Importantly, environmental factors such as allergens like dust mites, and *Staphylococcus aureus* which often colonizes AD skin, produce high levels of proteases which can further aggravate the barrier function.

GENETICS

It is now well established that AD is a genetically complex disease with a high familial occurrence. Multiple candidate genes have focused on adaptive and innate immune response genes, but recently the critical role of skin barrier dysfunction has been strongly supported by consistent replication that loss-of-function gene mutations in the epidermal structural protein filaggrin (FLG) is highly associated with the development of AD. FLG gene mutations are most frequently found in patients with more severe AD, early onset of this skin disease, enhanced systemic allergen sensitization. Aside from its role in maintaining the mechanical skin barrier in the stratum corneum, breakdown products of filaggrin also act as moisteurizing factors needed to maintain skin hydration. Therefore the lack of filaggrin can also contribute to dry skin.

The critical link between abnormal skin barrier in FLG deficient patients with AD and Th2 polarization may be explained in part by enhanced allergen penetration through the skin accompanied by increased production of thymic stromal lymphopoietin (TSLP). Allergens and/or microbes can trigger keratinocytes to produce TSLP, an IL-7 like cytokine which is overexpressed in AD epidermis and signal immature myeloid dendritic cells to induce development of Th2 cells by upregulating Ox-40L in the absence of IL-12 production. There is also an association between AD subjects with a history of eczema herpeticum (ADEH) and *Staphylococcus aureus* infection with FLG gene mutations. Mice that are deficient in the FLG gene have now been studied and found to spontaneously develop eczema. These mice also have an increased uptake of intact allergens through their skin. This defect in skin barrier

contributed to elevated systemic IgE sensitization and facilitated the initiation of allergic skin inflammation.

IMMUNOPATHOGENESIS

Despite the recent data highlighting skin barrier dysfunction as a major component of AD, substantial data still supports the immune response as critical for the development of AD. Indeed, primary T cell immunodeficiency disorders frequently have elevated serum IgE levels and eczematoid skin lesions which are cleared following successful bone marrow transplantation. In animal models, AD does not occur in the absence of T cells.

The immune response in AD is dependent on its duration of inflammation and the body compartment involved. The peripheral blood in most patients with AD is associated with elevated serum IgE and eosinophilia. Patients with increased IgE-mediated allergen sensitization involve nearly 80% of AD patients and are referred to as "extrinsic AD". Their skin homing (cutaneous lymphoid antigen or CLA+) T cells are predominantly T helper type 2 (Th2) immune response with allergen driven responses that lead to secretion of IL-4, IL-5 and IL-13 but only low levels of gamma interferon. This is important because IL-4 and IL-13 promote immunoglobulin isotype switching to IgE and IL-5 promote the development of eosinophils. Patients with "intrinsic AD" have no evidence of IgE allergen sensitization and lower levels of T cell expression of IL-4 and IL-13.

Nonlesional AD skin is not normal. It frequently manifests increased dryness and a greater irritant skin response than healthy controls. Unaffected AD skin contains a sparse perivascular T cell infiltrate, not usually seen in normal healthy skin. Analyses of biopsies from clinically unaffected skin of AD, as compared with normal non atopic skin, demonstrates an increased number of Th2 cells expressing IL-4 and IL-13, but not gamma interferon, mRNA.

Acute eczematous skin lesions present as intensely pruritic, excoriated erythematous papules. These skin lesions are characterized by marked epidermal intercellular edema (spongiosis). Antigen-presenting cells (APC) [e.g. Langerhans cells (LC), inflammatory dendritic epidermal cells (IDEC) as well as macrophages] in lesional skin bear IgE molecules. In the dermis of acute lesions, there is a marked infiltration of CD4+ activated T cells with a memory phenotype suggesting previous exposure to antigen.

The evolution of AD skin lesions is orchestrated by the local tissue expression of pro-inflammatory cytokines and chemokines. Cytokines such as tumor necrosis factor-alpha from resident cells (keratinocytes, mast cells, dendritic cells) binds to receptors on vascular endothelium,6 activating cellular signaling pathways that induce expression of vascular endothelial cell adhesion molecules. These events lead to adhesion of circulating inflammatory cells to the endothelium followed by extravasation and infiltration of these cells into the tissue, driven by their response to chemotactic gradients established by chemokines and chemotactic cytokines which emanate from sites of injury or infection. These molecules play a central role in defining the nature of the inflammatory infiltrate in AD. IL-16, a LC-derived chemoattractant cytokine for CD4+ T cells, has been found to be increased in acute AD skin lesions. CCL27 is highly upregulated in AD and preferentially attracts CLA+ T cells into the skin. As compared to psoriasis, the C-C chemokines, RANTES, and eotaxin are increased in AD skin lesions and likely contribute to the chemotaxis of CCR3-expressing eosinophils, macrophages and Th2 lymphocytes into AD skin. Selective recruitment of CCR4-expressing Th2 cells into AD skin may also be mediated by MDC and TARC, which are increased in AD.

Despite the strong associations between FLG null mutations and persistent AD, it should be noted that the same FLG mutations were first reported to occur in ichythyosis vulgaris, a dry, scaling skin condition not associated with significant skin inflammation. Furthermore a substantial number of patients with severe AD do not have FLG null mutations. Conversely there are normal individuals with FLG null mutations who have no evidence of skin disease. Most patients with FLG mutations also outgrow their AD by early adolesence. This suggests that additional factors are important in the development of clinical AD. Indeed, we have recently demonstrated that Th2 cytokines such as IL-4 and IL-13 can downregulate filaggrin production suggest that immune modulation plays a key role in driving skin barrier dysfunction. The mechanism by which IL-4 and IL-13 reduce filaggrin may be by decreasing the expression of S100A11, a calcium sensing keratinocyte differentiation protein.

The important role that Th2 cytokines play in the skin inflammatory response has been demonstrated in experimental models of allergen-induced allergic skin inflammation in mice with targeted deletions or over expression of these cytokines. In this regard, transgenic mice genetically engineered to overexpress IL-4 or IL-13 in their skin develop inflammatory pruritic skin lesions similar to AD, suggesting that local skin expression of Th2 cytokines plays a critical role in AD. Recent studies also suggest that the pruritus in AD may be caused by IL-31, which is also produced by Th2 cells. This is supported by the observation that mice genetically engineered to overexpress IL-31 become very itchy. Furthermore, in animal models of AD, anti-IL-31 reduces their scratching and a IL-31 gene variant is associated with the intrinsic form of AD.

In chronic AD skin lesions, there is evidence of tissue remodeling that are characterized by thickened plaques with increased markings (lichenification) and dry, fibrotic papules. An increased number of IgE-bearing LC and IDEC are observed in the epidermis, and macrophages dominate the dermal mononuclear cell infiltrate. Eosinophils also contribute to the inflammatory response, and T cells remain present, although in smaller numbers than seen in acute AD. Chronic AD skin lesions have significantly fewer IL-4 and IL-13 mRNA-expressing cells, but increased numbers of IL-5, GM-CSF, IL-12 and IFN-g mRNA-expressing cells than acute AD. Other factors that contribute to AD skin inflammation include the presence of IL-17 and IL-22 producing cells, IgE autoantibodies antibodies that react with epidermal antigens, and a lack of T regulatory cells needed to control chronic inflammation.

Dendritic cells have recently been demonstrated to be an important cell in bridging the innate and adaptive immune response in AD. These cells are armed with pattern-recognition receptors [such as toll-like receptors (TLR)] that sense the environment for danger signals and via various signal transduction pathways control the outcome of different T cell pathways. In AD, the presence of epidermal FceRI/IgE+ Langerhans cells is required to provoke eczematous skin lesions. It is thought that IgE armed dendritic cells in the skin facilitate allergen processing and activation of Th2 cells since bridging of FceRI with TLRs dampens interferon production. This is reinforced by the enhanced release of TSLP which acts on dendritic cells to initiate and perpeuate Th2 immune responses in AD.

IMMUNOLOGIC TRIGGERS

Food Allergy. Placebo-controlled, food challenge studies have shown that food allergens can induce eczematoid skin rashes in nearly 40% of children with moderate to severe AD. Children with food allergy generally have positive immediate skin tests or serum IgE directed to various foods particularly egg, milk, wheat, soy and peanut. Importantly, food allergen-specific T cells have been cloned from AD skin lesions providing direct evidence that foods can contribute to skin inflammation. In mouse models of AD, oral sensitization with foods results in the elicitation of eczematous skin lesions on food challenge.

Inhalant Allergy. Over the age of 3 years, food allergy is frequently outgrown but sensitization to inhalant allergens becomes more common. Pruritus and skin lesions can develop after intranasal or bronchial inhalation challenge with aeroallergens in sensitized AD patients. Epicutaneous application of aeroallergens [e.g. house dust mites (HDM), weeds, animal danders and molds] by atopy patch test on uninvolved skin of AD patients elicits eczematoid reactions in 30-50% of patients with AD. The isolation from AD skin lesions and allergen patch test sites of T cells which selectively respond to *Dermatophagoides pteronyssinus* (*Der p1*) and other aeroallergens supports the concept that immune responses in AD skin can be elicited by aeroallergens.

Infection. Most patients with AD are colonized with *S. aureus* and can have flares of their skin disease due to bacterial skin infection. Multiple mechanisms likely lead to exacerbation of skin disease from *S. aureus* but one important strategy is via secretion of toxins called superantigens which stimulate activation of T cells and macrophages. Most AD patients make specific IgE antibodies directed against staphylococcal superantigens; and these antibodies correlate with skin disease severity. Superantigens also induce corticosteroid resistance and may thereby make patients resistant to the anti-inflammatory actions of topical steroids.

Increased binding of *S. aureus* to AD skin is driven by underlying allergic skin inflammation. This is clinically supported by studies demonstrating that treatment with anti-inflammatory agents reduces *S. aureus* counts on atopic skin. In experimental animal models, *S. aureus* binding was significantly greater at skin sites with Th2-, as compared to Th1-, mediated skin inflammation due to IL-4 induced expression of fibronectin. AD skin has also been found to have a defect in S. aureus killing and this is thought to be due to a deficiency in antimicrobial peptides (e.g. cathelicidin and human beta defensins). Thus, once *S. aureus* binds to AD skin, inadequate host defense allows bacteria to colonize and grow. The lack of skin innate immune responses may predispose these patients to infection as well to fungi and viruses. This defect in generation of antimicrobial peptides is thought to be acquired this it is reversible when atopic keratinocytes are cultured in the absence of cytokines and occurs when skin is growth in the presence of IL-4 and IL-13. Importantly the abnormal antimicrobial response in AD skin can be reversed with antibodies that neutralize IL-4 and IL-13. There is also a strong association between serum IgE and eosinophilia with propensity to develop bacterial and viral skin infection including eczema herpeticum EH).

MANAGEMENT

Successful management of AD requires a multipronged approach. This includes the avoidance of irritants and allergens, including foods and aeroallergens, which can either induce the dermatitis or trigger the itch scratch cycle that results in AD. Skin hydration and use of emollients to repair the impaired skin barrier function is a key part of management. Although viral or fungal infection can trigger human AD, *S. aureus* colonization or infection is the most common cause of increased AD severity. In such patients a course of antibiotics, in combination with anti-inflammatory therapy, will lead to better control of skin disease. The key to successful long-term management of AD is the introduction of effective anti-inflammatory therapy such as topical steroids. In patients who do not respond to topical steroids consider the possibility of environmental factors, such as infection or allergens, which can induce steroid insensitivity. Topical calcineurin inhibitors are particularly useful in patients who are steroid insensitive as they act independent of glucocorticoid receptor or on areas of the body, such as the face, which are prone to steroid atrophy. In patients who are prone to relapse, maintenance topical anti-inflammatory therapy given as 2-3 times per week to skin areas prone to relapse, may be required to control skin disease.

In patients who are unresponsive to topical anti-inflammatory therapy, alternative systemic approaches should be considered. Ultraviolet (UV) light therapy can be a useful treatment modality for chronic recalcitrant AD. Oral cyclosporin A is a potent anti-inflammatory therapy which been demonstrated in multiple studies to be efficacious the treatment of severe, refractory AD, although primarily renal and liver toxicity limits its long term use. Antimetabolites including mycophenolate mofetil, a purine biosynthesis inhibitor, methotrexate, and azathiaprine have also been utilized for recalcitrant AD, although the potential for systemic toxicities restricts their use and requires close monitoring. Allergen-specific immunotherapy may also be useful in AD patients triggered by inhalant allergens such as dust mites. Other approaches that have been reported to be successful include recombinant human interferon gamma and early treatment with microbial probiotics. Case reports have suggested the potential usefulness of rituximab®, anti-IgE, TNF antagonists and intravenous immune globulin. There has also been considerable interest in the hypothesis that vitamin D deficiency is fueling the allergy epidemic. Indeed recent studies using oral vitamin D for treatment of AD have showed promising results in the augmentation of innate immune responses and clinical improvement of atopic skin disease.

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NOTES

ATOPIC DERMATITIS: THE VETERINARY PERSPECTIVE

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INTRODUCTION

The term "atopy" – from the Greek and literally translated as "strange disease" was introduced by Coca and Cooke in the 1920s to describe a familial hypersensitivity of humans that manifests as asthma or hay fever[1]. These workers also introduced the term "reagin", to describe the unusual type of antibody associated with the condition, which was (i) heat labile, and (ii) could be transferred to the skin of normal individuals – the so-called Prausnitz-Küstner (or PK) test[2]. Many years later this antibody was shown to belong to a hitherto-undescribed antibody class, which was named IgE[3]. Atopic dermatitis (AD) was added to this group of diseases later by Hill and Sulzberger[4], and through the ages, affected patients have often exhibited "the atopic march", where their disease commences with AD, and they later develop asthma or hay-fever. Although AD and asthma are usually associated with excessive production of IgE and exacerbated by environmental allergens, a subset exists, namely "intrinsic" in which allergen-specific IgE is not demonstrable.

In the search for suitable animal models of the atopic diseases, much emphasis has been placed on various mice models (e.g[5]). The great advantage of these, of course, is their cost-effectiveness, and the fact that specific genetic manipulations enable investigations of differing aspects in the pathogenesis. However, none of these develops a spontaneous disease when exposed to environmental conditions associated with modern living. It is only recently that the value of the canine model has become more widely recognized[6,7]. This presentation will discuss canine AD from the historical perspective and describe what is known about the pathogenesis, and the current therapeutic approaches. Finally, it will examine the question of whether the use of the term AD is justified in the cat.

THE HISTORY OF CANINE AD

The importance of allergy in skin diseases of dogs and cats became apparent to veterinarians in the USA in the 1930s. Schnelle, working at the Angel Memorial Hospital in Boston documented that 15% of all cases seen were accorded a diagnosis of "eczema". He also reported that 56.9% of all dogs with skin disease, and 26.6% of all cats similarly affected were deemed to be suffering from this condition[8]. Similar figures were reported from the clinics at Cornell University in Ithaca, New York[9].

Although it was generally believed that "eczema" was a manifestation of allergy, the exact nature of the inciting cause was controversial, with most emphasis being placed upon foods. In 1941, Wittich, a human allergist, described dog with perennial pruritus due to a food allergy that suffered seasonal hay-fever from a concomitant pollen allergy[10]. The dog was treated with an appropriate hypoallergenic diet and successfully hyposensitized with injections of allergenic extracts of the pollens to which sensitivity was shown. The association with IgE was further confirmed by demonstrating positive PK tests using both canine and human recipients.

In the 1960s there was considerable interest in canine ragweed pollenosis in the USA, and Roy Patterson, another human physician, developed a colony of atopic dogs suffering from the condition[11]. The dogs were reported as showing signs of hay-fever, and although they did not suffer from spontaneous asthma, the latter was inducible by insufflation with high concentrations of allergen. Furthermore, asthma was inducible in normal dogs following injection of atopic serum. Despite the fact that there were obvious

dermatological signs in addition to hay-fever-like signs, it was not thought to be truly analogous to AD of man. Instead it was termed "atopy", "atopic disease" or "allergic inhalant dermatitis"- the latter term in the mistaken belief that inhalation was the major route of access of allergen. This period saw the first detailed clinical description of the condition[12], which was followed by the identification and description of canine IgE only 6 years after that of its human counterpart[13], and of its association with mast cells in canine skin[14]. The development of tests for the measurement of canine allergen-specific IgE followed shortly therafter[15].

Thus throughout the 1960s and 1970s, the concept was of "an atopic disease" associated with allergen-specific IgE, and it was not until the 1980s and 1990s that the term canine AD came into common usage, and that the close similarities between this and the analogous human disorder were fully recognized[16,17].

DEFINITIONS

In the revised nomenclature for veterinary allergy[18], canine AD is defined as:

- A genetically predisposed inflammatory and pruritic allergic skin disease with characteristic clinical features associated with IgE antibodies most commonly directed against environmental allergens

However in a proportion of cases, variably estimated at between 10 and 30%, IgE antibodies to environmental allergens are not detectable either by intradermal testing or serology. The term "atopic-like dermatitis" is proposed for this condition, which appears analogous to intrinsic AD of man. The definition of atopic-like dermatitis is:

- An inflammatory and pruritic skin disease with clinical features identical to those seen in canine atopic dermatitis in which an IgE response to environmental allergens cannot be documented

The latter condition has attracted little attention. One preliminary study reported that the diagnosis was made in 21 out of 82 consecutive cases[19]. There was an apparent predilection for the French bulldog, and cases were significantly less responsive to cyclosporine than are cases of classical AD. As there is little information on this condition, the comments in this paper will be restricted to classical canine AD.

GENETIC FACTORS IN CANINE AD

A classical feature of the atopic diseases is that the trait is inherited, and breed predilections are to be expected. A study at the University of California compared the incidence to the base clinic population, with the Labrador retriever, golden retriever, West Highland white terrier, Chinese shar-pei, bull terrier, bichon frisé, Tibetan terrier and English springer spaniel all significantly over-represented, and mixed breeds protected[20]. The populations of teaching hospitals can be biased, however, and more reliable data is obtained when comparisons are made to the national population. The requirement in Switzerland for a national canine register has enabled such a study, in which the West Highland white terrier, boxer, French bulldog, bull terrier, Dalmatian, Vizsla and Basset hound were significantly over represented[21]. The heritability of AD in Labrador and golden retrievers bred as guide dogs for the blind was examined and found to be quite high at 0.47, again implying a strong genetic trait[22].

In contrast to mouse models which can result from single genetic defects, both human and canine AD are polygenic disorders with complex inheritance mechanisms. To study the possible association of genes with disease states, the "broad brush" approach employing microarray analysis of mRNA expression is a logical starting point that enables an assessment of large numbers of candidate genes. A recent study of canine AD identified 44 out of a total of 22,000 genes that were either over- or under-expressed in lesional or non-lesional skin of AD as compared to normals[23]. Many of these are related to barrier or immune function. Most notable was the S100 calcium-binding protein A8 (S100A8) which showed an almost 23-fold increase in lesional skin, but not in non-lesional skin. This protein, however, is increased in many other inflammatory disease states. Such analyses are indicative rather than quantitative, and a further study used quantitative real-time PCR to evaluate expression of 20 genes, 12 of which are known

to be dysregulated in human AD, and a further 8 candidate genes identified in the earlier study. Significant abnormalities were found in 11 of these. Again, genes concerned with immunological or barrier function were amongst those implicated, and some striking similarities were noted with results in man[24]. Interesting were positive correlations with severity scores as measured by CASEDI 03 in the case of S100A8, serum amyloid A1 (SAA-1) and plakophilin-2 (PKP2), with other abnormalities correlated with intradermal test reactivity. The caveats associated with these studies are many. Amongst them is the question of whether non-lesional skin is truly non-lesional, and also of whether the changes seen are specific for AD, or merely reflective of inflammation *per se.*

A later study by the same workers employed a genome-wide association analysis of disease-related single-nucleotide polymorphisms (SNPs) using a commercially available array covering 22,362 canine SNPs. The inbred nature of dogs means that this approach is far less complex and costly as compared to humans, where up to 500,000 SNP markers would be required to effect the same degree of coverage. Thirteen SNPs were found to be associated with canine AD. Some were limited to golden retrievers, whereas two were common across all of 8 breeds investigated[25].

These investigations are obviously of great importance in aiding a better understanding of the inheritance of this complex disorder, but an enormous body of work remains to be undertaken before a clear picture emerges.

THE DEVELOPMENT OF CANINE MODELS OF AD

Early attempts to develop colonies of atopic dogs for investigative purposes were either not predictably successful, or failed to find widespread usage in research[11,26]. More successful was the development of the high IgE-producing beagle model[27,28,29]. In this model, exposure to a slurry of dust mite antigen reproducibly induces a dermatitis clinically indistinguishable from AD. Although in this model the major route of access of allergen has been shown to be percutaneous, ingestion or inhalation of antigen induces dermatitis – albeit less dramatically[30]. More recently, the Maltese-Beagle inbred colony at North Carolina State University which initially showed spontaneous food hypersensitivity[31], has been shown to be another excellent model for AD, and animals again react to epicutaneous exposure to antigen[32].

RECENT RESEARCH ON THE IMMUNOPATHOGENESIS OF CANINE AD

(i) Investigations of clinical cases

Innate immunity

A number of defects in the innate defences have noted in human AD. Indeed a plausible case has been made that the condition could be primarily result from innate immune defects[33]. Only one study has addressed this in canine AD which showed that three 16 β-defensins were expressed in skin, namely cBD1, cBD103 and cBD107. Of these, cBD1 levels were significantly increased in non-lesional skin, and more so in lesional skin. In contrast, cBD103 was significantly underexpressed in non-lesional skin as compared with skin from healthy controls[34]. In confirmation of earlier studies, expression of the pro-inflammatory S100A8 was highly upregulated. Innate immunity is an area requiring further investigation.

Acquired immunity

Immunohistochemical studies have shown that the infiltrating cells in skin biopsies of spontaneous cases of canine AD comprise mast cells, dendritic antigen-presenting cells, T lymphocytes expressing γδ rather than αβ receptors with low numbers of neutrophils and eosinophils and rare B-lymphocytes[35]. Both CD4+ and CD8+ T cells are found in increased numbers, with a major increase in CD8+ cells in the epidermis along with microaggregates of eosinophils. There is proliferation of Langerhans cells armed with IgE[36], and similarly to man but in contrast to the mouse which has only a γ-chain, the Langerhans cell Fcε receptor (FcεR1) has been shown to possess α- and γ- but no β- chains[37]. Another careful study using both toluidine blue staining and enzyme immunohistochemical staining for chymase and tryptase failed to demonstrate significant differences in the mast cell density in the dermis of atopic and normal dogs, although significantly lower numbers stained for mast cell enzymes in samples from lesional and non-

lesional skin, implying selective degranulation[38]. Mast cell densities do vary with the site, with higher densities in the skin of the ear pinna and volar interdigital skin, which are both sites of predilection for AD in the dog[39].

Studies employing a non-quantitative reverse transcriptase PCR suggested that a clear Th2 polarisation was evident in some 25% of cases[40]. Two later studies employing clinical material using semi-quantitative methods yielded evidence of overespression of both Th1 (γIFN) and Th2 (IL-4) cytokines[41,42]. It was suggested that early lesions might be associated with a predominantly Th2 response which changed due to the ensuing chronicity and secondary infection to a Th1 response.

Interest has also focussed on the possible role of CC chemokines in AD. Thymus and activation regulated chemokine (TARC) is produced mainly from keratinocytes in response to inflammatory cytokines such as IL-1β, IFNγ and TNFα. It plays an important role in Th2 cell migration since its receptor (CCR4) is expressed selectively on Th2 cells. TARC was found to be expressed exclusively on lesional skin of atopic dogs, and was indeed associated with increased expression levels of IL-1β, IFN-γ and TNF-α[43]. In this study of chronic clinical AD, no increase in IL-4 was detectable. A later study by the same workers employing a monoclonal antibody to TARC confirmed keratinocytes in lesional skin of AD as the major source, and that its receptor (CCR4) was expressed on the infiltrating cells[44]. In another study of 7 chemokines, levels of the CCL28 expression in lesional skin was significantly increased, whereas those of CCL27 were significantly reduced[45].

(ii) Investigations using the beagle model

The Th2 vs Th1 issue was investigated further using atopy patch tests in the high-IgE beagle model in which it should be possible to separate out the acute and chronic phases. Amongst the Th2 cytokines, IL-6 and IL-13 were significantly increased and peaked at 24 hours[46]. Although IL-4 increased over 6-24 hrs, the increase was not significant. Amongst the Th1 cytokines, γIFN had a biphasic response with peaks at 6 hrs and 96 hrs with IL-18 gradually increasing through 96 hours. Thus although there is a pattern which is in general accord with that in man, results are not conclusive, and further studies are required. Levels of the chemokine TARC were again significantly increased, confirming an important role. In contrast there was no increase in another important chemokine – regulated on activation normal T dell expressed and secreted, or RANTES.

(iii) Investigations employing whole blood

The first of these assessed mRNA of IFNγ, IL-4, IL-5 and IL-10 in freshly isolated peripheral blood mononuclear cells from dogs with AD[47]. The results were inconclusive, with a reduction in IFNγ and an increase in IL-5, with no change in IL-4 and IL-10. The second study evaluated mRNA expression of IL-4, IL-13, IL-10 and TGFβ in the high IgE beagle model. IL-4, IL-13 were unchanged, but the levels of expression of the immunosuppressive cytokines IL-10 and TGFβ were reduced, which could imply aberrant regulatory T cell function[48].

BARRIER ABNORMALITIES IN CANINE AD

The critical role of epidermal barrier function in maintaining the integrity of the skin is well known, and abnormalities have long been recognized as a pivotal pathogenetic mechanism in human AD[49], with ceramides playing a major role. A vicious cycle ensues wherein bacterial colonization that is a feature of AD can lead to further lowering of epidermal ceramide levels through action of bacterial ceraminidases[50]. Therapeutic approaches to the management of human AD have thus centred on restoring barrier function, but is only relatively recently that attention has focussed on this aspect in dogs.

Transepidermal water loss

The integrity of barrier function is generally assessed by measurement of transepidermal water loss (TEWL). Although *in vitro* experiments cast some doubt on whether this provides an accurate assessment[51], the relationship between the two in dogs has recently been confirmed using tape stripping and gauging the barrier function by permeation of a fluorescent dye[52]. However TEWL measurement gives differing results depending on the precise technique used[53-55] (open vs closed chamber, site

variations, movement and presence or absence of hair) and careful validation is necessary. Despite these reservations, some important data has emerged from the beagle model confirming that TEWL is increased in sites prone to the development of AD prior to allergen exposure, and this is further increased in diseased skin when compared to age-matched normal beagles[56].

Analysis of surface lipids

A recent study has shown that the surface lipids of non-lesional skin of dogs with AD differ from those of normal dogs[57]. The levels of ceramides 1 and 9 were significantly decreased, whereas that of cholesterol was significantly increased, and ceramide/cholesterol ratio was significantly lower. The changes in ceramide 1 may be of especial significance, as this lipid is believed to be of particular importance in the assembly of the intercellular lipid lamellae[58]. A more recent study has confirmed that ceramides are reduced in both lesional and non-lesional skin of atopic dogs, and that this reduction is inversely correlated with the transepidermal water loss[59]. Another study has shown lowered mRNA expression of both Δ-5 and Δ-6 desaturase in both lesional and non-lesional skin of dogs with AD as compared with normal controls[60], although the precise relevance of this to barrier function is unclear.

Ultrastructural studies

Three ultrastructural studies have reported similar findings [61,62,63]. Instead of being organized into lamellae, the lipid deposits are reduced in both lesional and non-lesional skin and the deposits are heterogeneous, with widened intercellular spaces. In one study, delayed release of lamellar bodies was noted, and there was a sudden release of lamellar lipids upon allergen challenge[63]. Furthermore, filaggrin staining differed between atopic and normal dogs with finer granules and less intensity of staining in the former[7,64]. Of great interest was the observation that the staining in normal skin was reduced after dust mite exposure[63].

The special role of house dust mites

Dust mites and storage mites are the allergens most commonly implicated in canine AD. Two recent studies have investigated possible non-specific inflammatory effects on canine keratinocyte and fibroblast cultures. The first employed extracts of multiple species of mites, and measured cytokine levels in culture supernatants. Levels of IL-1 receptor antagonist (IL-1F3), growth related oncogene α and TGF-α were increased in keratinocyte cultures by one or more mite extracts, but there was no effect on a wide range of other cytokines[65]. Effects in fibroblast cultures was more dramatic, with significantly enhanced release of both IL-6 and IL-8. However whether penetration to the level of dermal fibroblasts could occur in atopic dogs is unclear.

The second study examined the effects of purified Der f 1. There was a significant increase over time in the expression of GM-CSF (which assists in Langerhans cell differentiation), the pro-inflammatory molecule TNF-α and of IL-8[66]. The latter recruits neutrophils, which are not a prominent feature in canine AD, and so the significance of this is unclear. Although levels of TARC were increased, there was no change over time.

House dust mite antigens, many of which are proteases, may well have other very important effects – particularly on barrier function[63]. Also, they have been shown to cleave CD23 from activated B cells, thus dysregulating IgE synthesis[67]. It is thus likely that they play a significant role in the pathogenesis, in addition to providing a rich source of allergen.

THE ROLE OF ADVERSE REACTIONS TO FOODS

Whilst adverse food reactions (AFR) – most of which are likely true hypersensitivity reactions – have long been considered part of AD in man, in veterinary medicine they have been generally considered as separate entities – whilst acknowledging that they are often important contributors, or flare factors. There are a number of reasons for this:

- Allergic reactions to foods are heterogeneous, and can involve different body systems (e.g. skin, GI tract) and have differing pathogeneses.
- Genetic susceptibilities are not identical. Although many breeds that show susceptibility to AD are also predisposed to AFRs, additional breeds – namely the German shepherd, pug and Rhodesian ridgeback are significantly predisposed[21].
- There are differences in the age of onset of clinical signs, with 16% of cases of AD presenting with clinical signs at < 1yr of age, as contrasted with 48% of cases of AFRs[21].
- Although the dermatological signs can be identical, a higher proportion of dogs with AD suffer from interdigital dermatitis as compared with those suffering from adverse food reactions[68].
- A significant proportion of cases presenting as AD that are either completely or partially responsive to dietary manipulation have concomitant gastrointestinal signs – although these may be mild[21,69].

It is the writers belief that consideration of the potential roles of environmental allergies and AFRs separately is more likely to result in the best clinical outcome. Nonetheless it is essential that all animals with perennial signs of AD be assessed for possible contributions from both.

Assessment of the possible role of adverse food reactions

Typically, the possible role of AFRs in cases of potential or confirmed canine AD are excluded by the failure to respond to a single diet containing a novel protein and carbohydrate based upon dietary history – either home prepared or commercial. Alternatively hydrolyzed diets are employed. It seems likely that this significantly underestimates the possible contributions of AFRs to these cases as:

- A true dietary history is difficult to obtain as the content of commercial diets is not always known.
- Cross-reacting allergens will be recognized by some, but not necessarily all dogs[70], and beef, lamb and milk often cross-react.
- Hydrolyzed diets are not, unfortunately the "gold standard" and some 10-20% of cases of AFR that are asymptomatic on individualized restricted protein diets will relapse on hydrolysate diets[71,72].
- In one study of 40 dogs who were asymptomatic on commercial single source protein diets, 21, 19 and 34 relapsed respectively when placed on chicken and rice, catfish and rice and venison and rice respectively – all of which were supposedly novel proteins[73].

Allergic reactions to foods in man are heterogeneous, both in their immunopathogenesis and clinical signs. It has not been generally acknowledged that the same is true for dogs. IgE-mediated reactions are probably in the minority in both species, and are characterized by a quick onset and relatively brisk response upon elimination of the offending food. In case of human AD, the choice of the most appropriate hypoallergenic diet is aided by a combination of IgE serology and patch testing[74]. Patch testing has not been assessed in the dog, and serology for food allergen-specific IgE is not viewed as helpful as a diagnostic aid. However until more is known about the immunopathogenesis of AFR in the dog, it would be logical to choose a hypoallergenic diet employing of a careful dietary history and serology – choosing dietary components to which no immunological reactivity is demonstrable. The question of home prepared versus commercial diets for diagnostic purposes is controversial, and robust data in favour of either is lacking, but a home prepared diet cannot be an inferior choice, with up to 6-8 weeks allowed for a response in chronic cases[75].

SECONDARY FACTORS IN CANINE AD

The role of staphylococci

The frequent occurrence of both bacterial overgrowth and overt pyoderma in atopic dogs is widely recognized, and indeed it is no exaggeration to say that "An atopic dog is a pyoderma waiting to happen". Enhanced adherence of *Staphylococci* to corneocytes of atopic dogs was first shown in 2000[76], and it was later shown that adherence was greater to corneocytes from inflamed skin, although some strain variability was reported[77]. In another study adherence was shown to correlate with pruritus scores, but there was no apparent correlation with the propensity to develop pyoderma within the atopic group, and antimicrobial therapy had no effect on adherence[78]. Four different strains of *Staph intermedius* (now

Staph pseudintermedius) were further compared to a human isolate of *Staph hominis*, and preferential adherence to corneocytes from atopic dogs was confirmed, with lack of adherence by *Staph hominis*[79]. Furthermore, atopic dogs have been shown to have a significantly higher level of carriage at carrier sites as compared to normals[80]. The propensity to develop antistaphyloccal IgE has also been shown[81], thus compounding the effects.

Similarly, the ubiquitous yeast *Mallasezia* is found in higher numbers on the skin of dogs with dermatological diseases[82], although precise data linking *Malassezia* overgrowth specifically to atopic dermatitis is lacking. Nonetheless, the fact that sera from atopic dogs have higher levels of *Malassezia*-specific IgE emphasizes the importance of this organism as a contributor to the total allergenic load[83].

The role of fleas

Atopic dogs have been shown to be significantly predisposed to the development of flea allergy dermatitis[84], thus justifying another statement to the effect that "An atopic dog is flea allergy dermatitis waiting to happen".

THERAPY OF CANINE AD – WHAT MIGHT THE FUTURE HOLD?

The therapy of canine AD involves targeting all three aspects of the pathogenesis – viz (i) the immunological abnormality, (ii) the defective barrier function, and (iii) the secondary factors. A complete discussion of the management is beyond this scope of this presentation, which will dwell on existing and potential approaches to (i) and (ii).

Correcting the immunological abnormalities

Immunotherapy

For most clinicians, this represents the cornerstone of the approach. Its efficacy was shown some years ago in the classical placebo controlled study of Willemse[85]. The importance of employing an allergen mix tailored to the individual case was re-emphasized in a more recent study in which immunotherapy employing *D farinae* only in multisensitive animals was ineffective[86]. Successful immunotherapy has been shown to be associated with an increase IFN-γ[87], and in Treg cell numbers and IL-10 production[88]. The dogs with AD were shown to have similar levels of Treg cells as normals, but these levels rose two-fold during immunotherapy.

Control of IgE levels using active or passive immunization

Recombinant chimeric anti-IgE monoclonal antibody is used therapeutically in the treatment of moderate to severe allergic asthma in man, and its use in an experimental situation in the dog was first reported 10 years ago[89]. However its use for human AD has proved less successful[90]. A major problem in the dog is that levels of IgE are some 100-fold greater than those in man[91], which naturally presents more of a challenge. However exciting adaptations of this approach in man have included targeting a unique antigen on IgE-producing B cells thus inducing apoptosis[90]. It is certain that we will hear more of this approach in veterinary medicine in the future. An alternative approach is that of inducing an auto-IgE response which has been attempted by immunisation with constructs containing segments of canine and opossum IgE thus rendering the latter antigenic[91]. In an experimental system, an anti-IgE response was indeed inducible, with IgE levels falling by a mean of 65% - clearly a promising approach[91].

Modulating the Th1/Th2 balance by other means

Administration of IFN is one way by which this can be achieved, and in a study employing recombinant canine IFN-γ in a randomized trial with diphenhydramine as the comparator, very significant efficacy was shown[92]. A more recent study has employed recombinant feline IFNω with cyclosporine as a comparator, and no significant difference was evident between the two[93].

The possibilities for the use of chimeric antibodies are limitless, and these can potentially be directed at a wide range of molecules in the immunopathogenic pathways. Thymic stromal lymphopoietin (TSLP) has been shown to be a major driver of the Th2 response. It is produced by mucosal cells and keratinocytes and acts on dendritic cells. In turn, these express OX40L, which interacts with OX40 on CD4+ T cells to drive the Th2 response, and inhibit the Th1 response[94]. TSLP can also be triggered by virus infections, which may account for the frequently association between infections and the onset of asthma in man. Schering Plough has patented the potential use of anti-TSLP in dogs as a treatment for atopic disease. A similar approach targeting OX40 should be just as viable.

Normalizing barrier function

This has long been the cornerstone in the management of human AD, but only recently have clinicians directed their attention to this in canine AD. The role of ceramides is of particular importance, and in an experimental canine model in which TEWL was increased following application of sodium lauryl sulphate, a 2% ceramide preparation and a 2% "intercellular lipid mixture" were both very effective in restoring barrier function[95]. In an ultrastructural study, application of a preparation containing ceramides, cholesterol and fatty acids to non-lesional skin of atopic dogs produced a near normalisation of the previously severely disorganized lamellar lipids[62]. Moreover, after six applications at 3-day intervals, there was normalization of the appearance of the lamellar bodies and of their extrusion process, implying absorption of the lipids and their use by the keratinocytes[62]. Ceramides are also contained in some shampoo formulations. It is unclear whether beneficial effects on pruritus that have been demonstrated in some instances result from an effect on TEWL, or from varying effects of other constituents[96].

Nutritional supplementation also has a potential role. In a recent study, 5 components were selected following *in vitro* studies that assessed their effect on lipid synthesis by cultured keratinocytes. They were then added to the diet of normal dogs, and after 9 weeks of supplementation significantly reduced TEWL was demonstrated[97]. It is intriguing to speculate on whether the demonstrated benefits of specially formulated diets on the severity of canine AD may in part be a result of improved barrier function[98].

DO CATS SUFFER FROM AD?

The relative safety of corticosteroids in cats has meant that little work has been done on feline allergic diseases. Furthermore, the limited spectrum of reaction patterns in feline skin, and the fact that any one of these can result from a number of allergic and non-allergic etiologies has frustrated investigators. However when we consider the original definitions of "atopy", there is increasing evidence that cats may be considered as indeed suffering from atopic diseases, in that allergic asthma, AD triggered by environmental allergens and IgE-mediated adverse food reactions are encountered[99].

Feline AD was first reported by Reedy in 1982[100], and the manifestations are now generally considered to include miliary dermatitis, head and neck pruritus, eosinophilic granuloma complex and self-induced ("barbered") alopecia. As in dogs, the diagnosis is made by demonstration of compatible clinical signs, and exclusion of any other possible explanation for these signs.

Is there evidence that the disease(s) are inherited?

So far, evidence is sparse, with only one report of an AD-like condition in three littermates[101]. Another report details concomitant dermatitis and enteritis in 8/26 inbred cats of Hungarian origin[102], ascribed to an AFR.

Is there an association with IgE?
Feline IgE has been characterized, and both monoclonal and polyclonal antisera developed for its detection[103,104]. The human Fcε receptor also been employed in serological assays. Although earlier studies employing the latter failed to show differences in allergen-specific IgE levels between sera from allergic and normal cats[105], a recent study employing a monoclonal antibody demonstrated significantly higher levels of IgE specific for D farinae in suspected atopic cats as compared to age matched normals[104]. Interestingly, as is the case in dogs, house dust mite-specific IgE is commonly found in normal cats, but virtually absent from cats reared in laboratory conditions[103,104]. Although there have been no placebo controlled studies of immunotherapy, one open study suggested an efficacy rate of 50-75%[106]. In common with canine AD, the condition responds well to cyclosporine A[107,108].

What does immunohistochemistry and/or PRC studies of affected skin tell us?
Results are mostly compatible with an atopic disease, although not conclusively so. Immunohistochemistry has revealed proliferation of CD1a Langerhans cells in lesional skin[109], increased numbers of CD4+ T cells, with a lesser increase in CD8+ cells in both lesional and non-lesional skin[110], and significantly higher numbers of IL-4 producing cells in both lesional and non-lesional skin as compared to normals[111]. Similar results were obtained following atopy patch testing of clinical cases[112]. In contrast, quantitative real-time PCR measurement failed to reveal differences in mRNA expression between affected and normal cats[113].

Barrier function
There have been no studies on skin barrier function in cats with putative AD.

Can feline asthma be considered an atopic disease?
Asthma is commonly encountered in feline practice[114,115], but again, there has been little in the way of clinical research and long-acting injectable corticosteroids are routinely employed. An association with IgE has been recently shown, and importantly some animals suffer from concomitant skin disease[116]. An experimental model has been developed, and the efficacy of rush immunotherapy demonstrated[117,118]. Immunotherapy and allergen avoidance has also been demonstrated to be affective in an open study[119].

Conclusions
There is moderately strong data justifying the use of the term AD for some cases of allergic skin disease in cats. The fact also that cats suffer from an allergic asthma, sometimes with concomitant skin disease, lends further weight to the contention that cats do indeed suffer from atopic diseases.

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NOTES

WHAT'S NEW WITH MAST CELL TUMORS

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OVERVIEW

Tumors of the skin and subcutaneous tissue are the most common tumors affecting dogs, accounting for approximately one-third of all tumors encountered in the species. While many tumor types occur in this site, mast cell tumors head the listing of the ten most frequent non-lymphoid cutaneous tumors in the dog based on over 6000 cases in 4 continents and the 5 most frequent in the cat based over 1000 cases are presented in ***table 1 & 2*** respectively.

Table 1: Frequency (Percentage) of the Top Ten Non-lymphoid Cutaneous Neoplasms in the Dog (N = 6,282)[1]

Neoplasm	Percent of Cases
Mast cell tumor	18.8
Hepatoid (perianal sebaceous) adenoma/carcinoma	10.1
Lipoma	7.1
Sebaceous hyperplasia/adenoma	7.1
Histiocytoma	6.7
Squamous cell carcinoma	6.2
Melanoma	6.2
Fibrosarcoma	6.1
Basal cell tumor	4.6
Hemangiopericytoma#	4.4

Many refer to these now as nerve-sheath tumors

Table 2: Frequency (Percentage) of Top Five Cutaneous Neoplasms in the Cat (N = 1,155)[1]

Neoplasm	Percent of Cases
Basal cell tumor	19.7
Mast cell tumor	17.4
Fibrosarcoma	17.4
Squamous cell carcinoma	11.4[#]
Sebaceous hyperplasia/adenoma	3.1

- May be misleading as one of the surveys did not include ear tumors, a common site for squamous cell carcinoma.

Older names for MCT include mast cell sarcoma and histiocytic mastocytoma. Mast Cell tumors (MCT) are primarily a disease of older dogs and most occur in mixed breeds; however, Boxers, Boston Terriers, Labrador Retrievers, Beagles, and Schnauzers have all been reported to be at higher risk. While Boxers are at increased risk for MCT development, they more commonly develop the histologically well-differentiated form of the disease that carries a more favorable prognosis. Two distinct forms of cutaneous MCT in the cat have been reported: (1) The more typical mastocytic MCT, histologically similar to MCT in dogs and (2) The less common histiocytic MCT, with morphologic features characteristic of histiocytic mast cells. The etiology of MCT in the dog and cat is still unclear although familial associations and several molecular and genetic alterations are documented to play a role in their development and biological behavior. On rare occasions MCT have been associated with chronic inflammation or the application of skin irritants. Mutations of the tumor suppressor gene p53 and the

proto-oncogene c-kit have been found in approximately 50% of canine mast cell tumors and their frequency appears to correlate with histologic grade and biological behavior. No association with feline leukemia virus (FeLV), feline immunodeficiency virus (FIV) or feline infectious peritonitis (FIP) has been reported in the cat. A genetic predisposition has been proposed due to the high incidence of MCT in the Siamese breed.

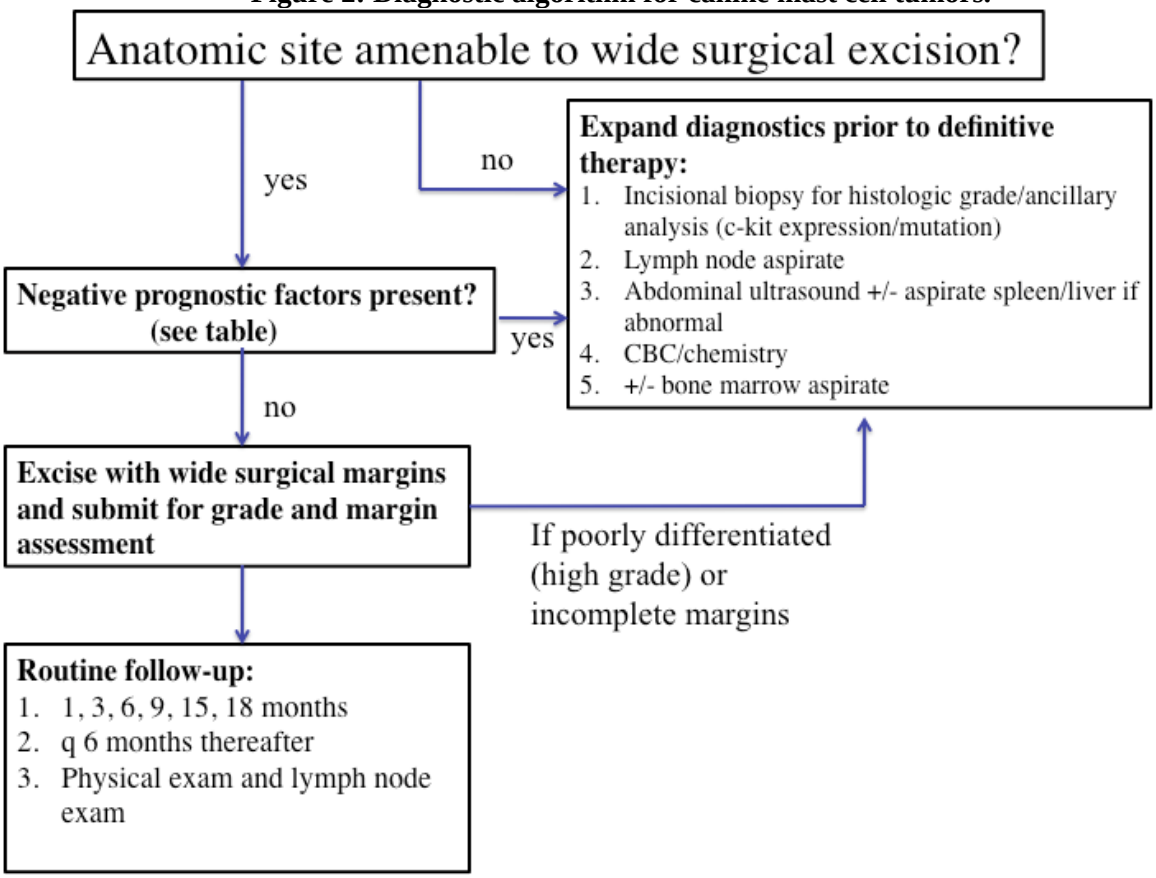
HISTORY AND CLINICAL SIGNS. The history and clinical signs of dogs and cats with MCT is complicated by signs attributable to release of histamine, heparin and other vasoactive amines from the MCT granules. These include coagulation disorders, gastrointestinal ulceration (with related signs of vomiting [possibly with blood] anorexia, melena, and abdominal pain), altered smooth muscle tone, hypotensive shock, and anaphylactoid reactions. Occasionally, mechanical manipulation during examination of the tumor results in degranulation and subsequent erythema and wheal formation in surrounding tissues. This phenomenon has been referred to as "Darier's sign". In dogs, MCT are most commonly found on the trunk; tumors on the limbs account for only one-quarter of all sites and lesions are least common on the head and neck. This is in contrast to cats where the head and neck is the most common site for MCT. A visceral form of MCT, often referred to as disseminated mastocytosis, can also occur.

DIAGNOSTIC WORK-UP. Mast cell tumors are initially diagnosed on the basis of fine-needle aspiration (FNA) cytology. Mast cells appear as small to medium-sized round cells with abundant, small, uniform cytoplasmic granules that stain purplish red (metachromatic). A small percentage of MCT have granules that do not stain readily, giving them an epithelial, 'fried egg', or macrophage-like appearance. In these cases, histological assessment is necessary for diagnosis. The extent of ancillary diagnostic work-up following FNA cytological diagnosis is based on the presence or absence of the negative prognostic factors (***Table 3***) for MCT in dogs (e.g., histologic grade, mitotic index, c-kit analyisi, proliferation indices, clinical signs, location).[2-8] The authors diagnostic algorithm for MCT in dogs is presented in ***figure 1***. There is some degree of controversy among veterinary oncologists as to how extensive clinical and clinicopathalogic assessments should be taken; however, it can be said that for the majority of cutaneous mast cells in dogs that are of low or intermediate grade and in a location conducive to wide excision, a minimalist approach can be taken.

Table 3: Prognostic factors associated with MCT in dogs.[1]

Factor	Comments
Location	Mucous membrane sites worse prognosis; other historically poor sites are controversial (inguinal and perineal)
Stage	LN (if not adequately controlled), distant metastasis
Recurrence	Recurrent tumors worse prognosis
Surgical margins	If incomplete, worse if not adequately controlled.
Histologic grade	Undifferentiated worse prognosis
Mitotic index	Index > 5 per 10 high power fields – worse prognosis
AgNOR	Higher count, worse prognosis
c-Kit mutations	Worse prognosis
p53 mutations	Worse prognosis
Clinical signs	More likely to be high grade or advanced stage –worse prognosis
Recent rapid growth	Poorer prognosis
Multiple cutaneous masses	Not prognostic

Figure 2: Diagnostic algorithm for canine mast cell tumors.

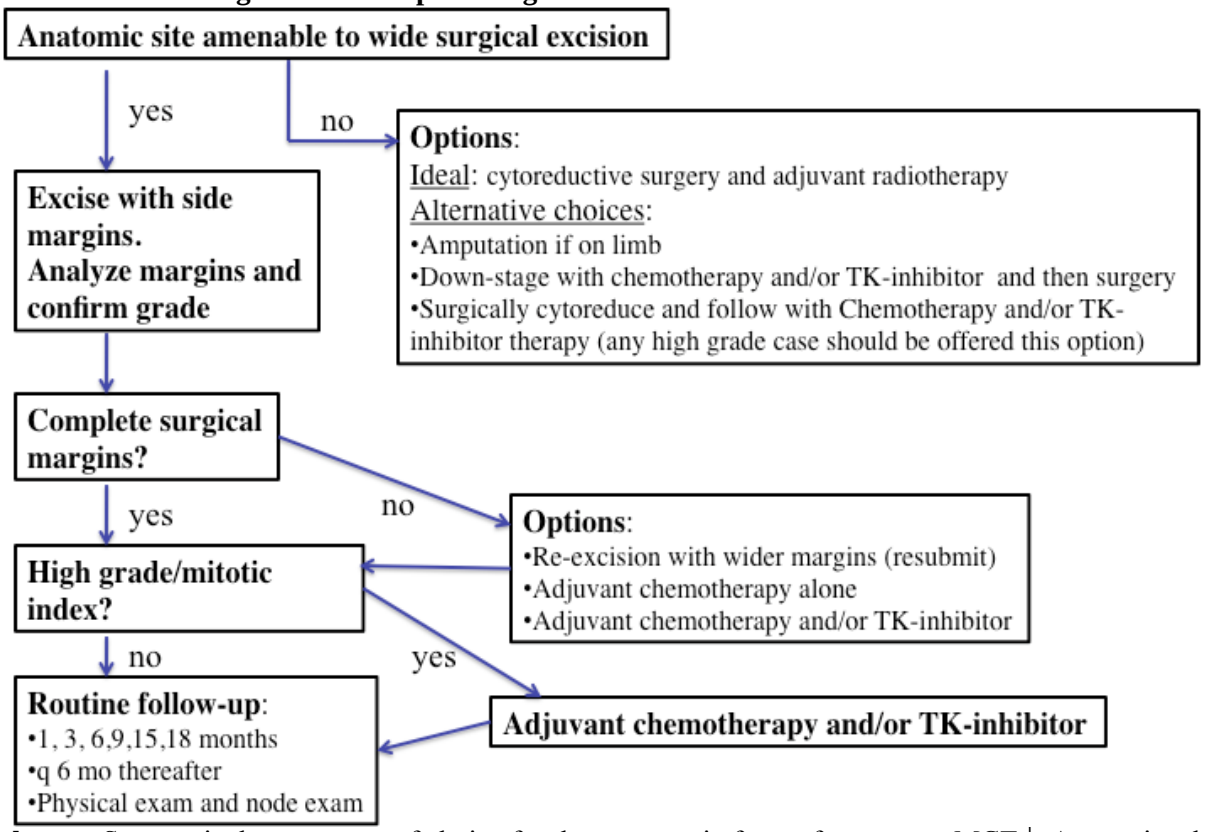


THERAPEUTIC APPROACH BY SPECIES. **In the dog**. Treatment decisions are also predicated on the presence or absence of negative prognostic factors and on the clinical stage of disease (***Figure 2***). Surgical removal is the treatment of choice for local MCT disease. Grade II and III lesions warrant aggressive local resection, obtaining 2 cm lateral margins and one additional mesonchymal tissue margin deep to what the tumor touches grossly. In certain areas, this type of resection will require some type of reconstructive procedure, or possibly a regional resection to be complete. Normal tissue margins should always be identified after removal so that the pathologist can assess the completeness of resection. In cases of incomplete resection, revision surgery should be considered first if feasible. For revisions, new margins are obtained as described above surrounding the old scar. Complete surgical resection for dogs with no evidence of metastasis will result in upwards of 90% local control for grade 1 & 2 tumors. For incomplete resection that is not amendable to surgery, radiation therapy to the site can be successful. Fractionated doses of approximately 48 Gy or higher have resulted in 80-90% 3 year control rates for grade 1 & 2 tumors. Alternatively, if radiation is not an option due to availability or cost, time to recurrence can be greatly extended with adjuvant chemotherapy (see below). For cases that are not amenable to surgical resection, several options exist. Neoadjuvant prednisone or chemotherapy may make an otherwise unresectable lesion resectable following chemotherapy. The second option is external beam radiotherapy alone; however, in the gross disease setting this results in one-year control rates of only 50%. Recently, course fraction radiation protocols (3 or 4 weekly 8 Gy fractions) have, anecdotally, resulted in local responses lasting months to even a year or longer. The third, and in the authors opinion, the ideal option for low or intermediate grade MCT in areas where wide surgical excision is not possible is a combination of surgery and radiotherapy. The complimentary use of surgery to achieve clinical stage 0 disease (i.e. microscopically incomplete margins) and external beam radiotherapy is associated with long term control (two-year control rates of 85 to 95%) of low or intermediate grade differentiation. Some authors advocate prophylactic irradiation of cytologically negative regional lymph nodes however; definitive evidence of a survival advantage associated with this practice is currently lacking. Unfortunately, dogs with undifferentiated tumors do not fare as well, with the majority developing distant metastasis within 4 – 6 months of therapy.

The management of biologically high grade MCT remains more difficult. Systemic adjuvant therapy should be offered in such cases in an attempt to decrease the likelihood of systemic involvement, or at least potentially improve progression-free intervals. Corticosteroids such as prednisone have been reported for many years in primarily preclinical or anecdotal settings to be of some benefit. In the authors practice, patients with poorly differentiated MCT receive prednisone and vinblastine as a first line. In this protocol, vinblastine (2.3 – 2.6 mg/m^2, IV) is given weekly for 5 consecutive weeks. Several other vinblastine based protocols have also been published. Prednisone is given at 1 mg/kg, P.O., once daily for 2 weeks, then decreased to 0.5 mg/kg daily for 3 additional weeks before being tapered off. Responses can also be expected with protocols using CCNU (Lomustine, 60 - 70 mg/m2 Q 3 weeks); however, CCNUs toxicity profile (i.e., thrombocytopenia, hepatic toxicity) temper its use in our hands to those cases having failed vinblastine or if oral chemotherapy is preferred. Other chemotherapy agents have shown activity including cyclophosphamide, hydroxyurea, chlorambucil, and vinorelbine.[9-13] Recently, tyrosine kinase receptor antagonist, in particular C-kit inhibitors (Palladia® & Masivet®) have been investigated and licensed for the treatment of dogs with MCT.[14-15] Early results are promising and several TK-inhibitors are under field trial investigation at this time for use in veterinary practice for several tumor types. Current recommendations for these agents will be discussed.

Ancillary therapy for the systemic effects of MCT related to degranulation is sometimes, but not always recommended. Blocking all or some of the effects of histamine release can be accomplished by administering the H_1 blocker diphenhydramine (2 - 4 mg/kg PO BID) and the H_2 blockers famotidine and omeprazole.

Figure 2: Therapeutic algorithm for canine mast cell tumors



In the cat. Surgery is the treatment of choice for the mastocytic form of cutaneous MCT.[1] As previously discussed, most are behaviorally benign and wide surgical margins may not be as critical as in the dog. This is fortunate, as most occur on the head where such margins would be difficult to achieve. Frequency of local recurrence and systemic spread vary widely in the literature. Local recurrence and frequency of systemic spread have been reported to occur in 5 – 10 % of cases. For histologically anaplastic (i.e. diffuse) mastocytic tumors, a more aggressive approach similar to that utilized for canine MCT may be prudent, as higher rates of recurrence and metastasis are associated with this type. Little is known about

the effectiveness of adjunctive therapy for cutaneous MCT in the cat. Visceral MCT of cats occurs in two forms; the splenic (hemolymphatic) or gastrointestinal form. Cats with the splenic form present with massive splenomegaly, oft peritoneal effusion and gastrointestinal signs (i.e. vomiting). Peripheral mastocytosis with this form is common. Surprisingly, long-term survival appears to be the norm following splenectomy, even in the face of peripheral mastocytosis (median survivals =18 months). The gastrointestinal form of visceral MCT is quite different in behavior than the splenic form. None have been reported to have circulating mast cells. A grave prognosis is prudent based on the high rate of metastasis. No data is available on surgical resection or chemotherapeutic intervention.

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NOTES

WHAT'S NEW WITH MELANOCYTIC TUMORS (MELANOCYTOMA, MALIGNANT MELANOMA)

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INTRODUCTION

Tumors of melanocytes and melanoblasts are relatively common skin tumors in the dog accounting for between 5 and 7% of all canine skin tumors.[1] They are a rare tumor in the cat, accounting for between 0.8 and 2.7% of all feline skin tumors. Melanocytic tumors are most common in older dogs (average age 9 years) with darkly pigmented skin although the literature varies in terms of which breeds are at risk. Early reports mention a male preponderance for tumor development, however, more recent reports do not support this. Melanocytic tumors are also more common in older cats (average age of 10 - 12 years), and no sex or breed predilection is known. The etiology of melanocytic tumors in dogs and cats is largely unknown; however, several investigations of the molecular and genetic basis of melanoma have previously been reviewed elsewhere (***Table 1***). Because they arise primarily in areas of haired skin or in the oral cavity, the causative association with ionizing sunlight is not a factor in these species. Cutaneous melanomas in the dog can be behaviorally benign or malignant and can occur anywhere on the body.

CLINICAL PRESENTATION/DIAGNOSIS

Melanocytic tumors can usually be diagnosed by simple fine needle aspirate cytology; however histology is important to determine malignant potential. Benign forms are often referred to as melanocytic nevus, a term which in its purest sense implies any congenital, melanin-pigmented lesion and this term has been frowned upon in the veterinary literature. Benign melanomas are typically well defined, deeply pigmented, less than 2 cm in diameter, dome shaped, firm, broad based but mobile over underlying tissue. Behaviorally malignant melanomas tend to grow rapidly, can be greater than 2 cm, and are often ulcerated and have irregular borders. A summary of factors known to be prognostic for cutaneous melanoma in dogs is presented in ***table 2***. Over 85% of melanomas in dogs arising from haired-skin are associated with benign behavior. The majority of oral and mucocutaneous junction melanomas (except eyelid), and approximately one-half of melanomas arising in the nail bed are behaviorally malignant.[1,2] Most melanomas can be diagnosed cytologically; however , tissue biopsy is recommended as the histologic criteria of mitotic index is highly predictive (approximately 90% accurate) of degree of malignancy. A mitotic rate of less than 3 per 10 high power field is strongly associated with benign behavior. Additionally, highly proliferative melanomas, as measured by Ki67 or PCNA immunohistochemical analysis has been shown to be associated with a more malignant course; however, these more advanced procedures offer little more predictive value than mitotic rate. The presence of mast cells at the edge and within melanoma tissue has also been associated with a more aggressive biology.[3] Tumors that are grossly infiltrative to underlying structures are also know to represent a more malignant variety. Breed has been reported to be of prognostic significance; more than 75% of melanoma in Doberman pinchers and miniature schnauzers are behaviorally benign while 85% of melanomas in miniature poodles are behaviorally malignant. Analysis of DNA ploidy with flow-cytometric analysis strongly correlates with degree of malignancy for melanoma in the dog. However, it was no more predictive than routine light-microscopy and therefore is not cost-effective. Again, this relatively time consuming technique is not clinically useful as a prognostic indices, however, it may have utility in differentiating amelanotic melanomas from other poorly differentiated tumors. Expression of p53 and several other tumor suppressor genes have been evaluated in a limited basis in canine and feline melanomas, and do not appear to have significant predictive value for outcome.

Amelanotic melanomas can occur at cutaneous sites, although they are much more common in the oral cavity of dogs. Special histochemical and immunohistochemical stains may be helpful in diagnosing these agranular variants.[1] Immunohistochemical stains have also been applied in the differentiation of melanomas and pigmented basal cell tumors in cats. Balloon, signet-ring, clear-cell melanocytoma-acanthoma, and pilar neurocristic histologic variants of cutaneous melanoma have been reported. The prognostic significance of these rare tumors is largely unknown, however no recurrence or metastasis developed following excision of 4 clear cell melanomas.

In the cat, melanogenic tumors can also be benign or malignant. While they can be induced experimentally with the feline sarcoma virus, it is unlikely to be associated with clinically observed cases. The majority involve the head (e.g., nose, pinna), and less commonly involve the extremities. Most are ocular or on the eyelid. Non-ocular melanomas in cats are similar in appearance to those in the dog, however, histologic assessment of malignancy does not appear to predict clinical outcome as in the dog. In general, ocular melanoma is behaviorally more malignant than oral melanoma in the species, and dermal melanomas can have a benign or a malignant clinical course.

THERAPY

The treatment of choice for local cutaneous melanoma in both the cat and dog is surgical excision. Those tumors in dogs with benign prognostic criteria (***Table 2***) carry an excellent prognosis following complete excision. Prognosis for those with malignant criteria is guarded as metastatic rates between 30 and 75% have been reported. In the cat, prognosis is fair for non-ocular dermal melanomas as recurrence and metastatic rates of 5 to 50% have been reported. Alternatives to blade excision for local disease include radiotherapy, local hyperthermia, electrochemotherapy, intralesional cisplatin/carboplatin, and photodynamic therapy.[1,4] Coarse fraction radiotherapy has been used with success for local control of oral melanomas in dogs and it is likely to be beneficial for dermal melanomas where surgery is not an option. However, most dogs with malignant disease will succumb to systemic spread. Response to hyperthermia/intralesional cisplatin and photodynamic therapy appear to be short-lived.

Systemic chemotherapy for malignant melanoma in the dog has shown little promise in the veterinary and human literature. Agents which have been investigated on a limited bases, and primarily for oral melanoma, include mitoxantrone, doxorubicin, and. In general, response rates are low and durations of response have been short-lived. Because of the absence of efficacious chemotherapeutic regimens for metastatic or unresectable melanomas, several novel therapeutic modalities have and are being investigated. These include methods of enhancing immunosurvelance (e.g. tumor vaccines) and immune mediated killing of tumor cells, as well as several techniques to target the tumor cells at the molecular level. Many of these studies were conducted as translational modeling for therapies in both veterinary and physician-based oncology and while most were for oral malignant melanoma, responders with cutaneous or digital melanoma have also been reported. Immunomodulation therapy is currently an active area of research for malignant melanoma in both physician- based and veterinary oncology.

IMMUNOMODULATORY THERAPY FOR MALIGNANT MELANOMA

Non-specific Immunomodulation: The classic example in the veterinary literature is the use of L-MTP-PE, a liposome encapsulated bacterial cell wall component. Macrophages and monocytes activated by muramyl tripeptide (MTP) acquire the ability to recognize and destroy neoplastic cells by a variety of mechanisms. L-MTP-PE not only increases monocyte tumoricidal activity but also causes increases in plasma concentrations of tumor necrosis factor-alpha (TNFa), and interleukin-6 (IL-6) among other cytokines. We have used L-MTP-PE immunotherapy In dogs with osteosarcoma (OSA), hemangiosarcoma, and malignant melanoma in randomized blinded trials involving several hundred dogs and L-MTP-PE significantly prolongs the metastasis free intervals and overall survival times when given alone or following chemotherapy in OSA and hemangiosarcoma and has shown some activity in dogs

with malignant melanoma. Negotiations are currently underway for licensing of L-MTP-PE for use in dogs. We are currently investigation this product in combination with radiation therapy and more specific immunotherapy using anti-cancer vaccines (see subsequent). Other non-specific immune based therapies under study in veterinary medicine include the use of nonsteroidal anti-inflammatory drugs, Fas ligand and CD40 ligand immunotherapy.[1,5,6]

ANTI-CANCER VACCINES[7]

Vaccines have been used to prevent infectious disease for over two hundred years. Ironically, their widespread use in veterinary medicine is one of the reasons cancer is such an important disease in veterinary practice owing to the extended life-span of the patients under our care. In the case of infectious disease, the immune system must recognize and attack non-self antigens that are foreign. This is in contrast to cancer vaccines where the immune system must recognize and attack "self" antigens that are derived from the host and are likely present on normal host tissues. Since the immune system has evolved through the millennia to become "tolerant" of self (i.e., so called *anergy*) and spare normal tissues (otherwise, immune mediated disease would be rampant), methods of safely breaking "self-tolerance" are important to the development of anti-cancer vaccines. Our laboratory and others have investigated several novel vaccine approaches to treating malignant melanoma. Whole-cell vaccines approaches have been used extensively in veterinary clinical trials and have the advantage of simplicity, as well as not requiring prior knowledge of which antigens are important as all potential antigens present in the tumor cell are used. Additionally, whole cell vaccines can be genetically altered (*transfected*) to produce adjuvant peptides (e.g., GM-CSF) at the site of vaccination, such that both antigen and adjuvant are presented together. Whole cells can also be programmed to over-express tumor-associated antigens (TAAs) in the hopes of eliciting a more robust immune response.[8] Other approaches include strategies to elicit immune responses to xenogeneic antigens (e.g., xenogeneic gp100 and tyrosinase) in the hope of creating cross-reaction between the xenogeneic homologs and self-molecules, breaking tolerance and ultimately resulting in a clinically relevant immune response. These strategies have been investigated either through genetically engineered whole-cell approaches, or through the use of xenogeneic DNA vaccines; that is, specific sequences of DNA injected into the host are decoded and the message translated into specific antigens that professional antigen presenting cells utilize, resulting in an antigen-specific immune response. This strategy has been employed by Phil Bergman's group at the Animal Medical Center in dogs with melanoma with some success and this vaccine currently has provisional approval in the USA under a USDA license.[9] Other active areas of cancer vaccine development include disialoganglioside (GD3) vaccines, dendritic cell vaccines and combining vaccine strategies with radiation (radio-immunotherapy) and chemotherapy to take advantage of the so-called 'abscopal' effect as well as vaccine strategies designed to target the "normal" host stroma and vasculature that support tumors, rather than the tumor itself.[10,11,12] Examples of current and future investigations of these strategies in pet dogs will be presented.

SUBUNGUAL (NAIL BED) MELANOCYTIC TUMORS

Primary subungual tumors are common in the dog and rare in the cat. A number of large case compilations exist on tumors of this location.[1] Approximately one-third to one half of subungual tumors in the dog are squamous cell carcinoma (SCC), followed in frequency by malignant melanoma, osteosarcoma , various soft tissue sarcomas (fibrosarcoma, neurofibrosarcoma) and mast cell tumors. While no breed or gender predilection is reported, in one large compilation 25% of dogs with malignant nail bed tumors had a black hair coat. In the cat, while primary nail-bed tumors are rare (almost always SCC), metastatic nail bed tumors are more common and are usually metastatic lesions of carcinomas from other sites.

Typical presenting signs in dogs and cats with subungual tumors are the presence of a mass, lameness, and ulceration. The associated nail may be fractured or absent altogether.Radiographs of the affected digit should be a routine part of the work-up for nail-bed disease as approximately 75% of primary digital tumors result in local bone lysis. If a malignancy is suspected or multiple digits are involved, thoracic radiographs are also indicated, particularly in the cat. While benign or infectious processes (pododermatitis) of the digit can result in local bone lysis, it is much less likely to occur. Subungual tumors are often secondarily infected and initially misdiagnosed as chronic paronychia or osteomyelitis. Prolonged histories prior to diagnosis may result.

Subungual melanomas are often malignant in the dog. Approximately one-third to one-half of melanomas originating in the nail bed will develop distant metastasis to lymph node, lung and other systemic sites. Digital amputation will usually control local disease (local recurrence rates of 30% can be expected), however, approximately half of dogs will die due to distant metastasis. It would appear that effective adjuvant systemic therapy is necessary for the majority of cases, however, as previously discussed, no consistent adjuvants exist for malignant melanoma and a fair to guarded prognosis is warranted.

Table 1: Molecular and Genetic Factors associated with cutaneous tumors in Dogs and cats.

p53	Altered expression of this gene that is crucial for DNA integrity reported in several tumor types in dogs and cats. P53 mutations documented in feline cutaneous tumors.
Metallothionein Expression	Can disrupt p53 and implicated in canine and feline melanocytic tumors.
RB-1	This cell cycle regulation gene is implicated in melanocytic tumors.
Cell survival and proliferation factors	Bax/Bcl-2 expression implicated in feline basal cell tumors. Relative apoptosis and proliferation rates altered in several cutaneous tumors.
Cyclin Kinase Inhibitors	p21/*waf-1*, p27/*kip1*, p16/*ink-4a* implicated in canine melanocytic tumors
PTEN	Altered expression of this important tumor suppressor gene implicated in canine melanoma
N-*ras*	Mutation in this protooncogene implicated in canine melanoma
Angiogenic factors	Angiogenic factors (VEGF) implicated and phenotypic characteristics (integrin expression and vascular density) documented in canine squamous cell carcinoma and melanoma
Telomere biology	Alterations in expression of telomerase in many cutaneous tumors
Heat shock proteins	These proteins implicated in control of growth and differentiation of several neoplasm in people and have been reported to be overexpressed in canine cutaneous epithelial tumors.

Table 2: Known or suspected prognostic factors for malignant melanoma in the dog

Factor	Comment
Location	Tumors arising from haired skin are generally benign. Tumors arising from mucocutaneous junctions (except eyelid), nail bed, and oral lesions are generally malignant.
Histology	Histologic criteria of malignant versus benign is very predictive of biologic behavior. Determination based primarily on the mitotic index; however, lymphatic invasion, microvascular density, presence of mast cells may also be predictive.
Breed	More likely benign in Doberman pinscher and miniature schnauzer, more likely malignant in miniature poodle.
Tumor cell proliferation rate	Histochemical and immunohistochemical (e.g., MIB-1/Ki67, PCNA) techniques that measure proliferation shown to be prognostic. Only modestly more predictive than simple histology and mitotic index.
DNA ploidy	Prominent G2/M peaks predictive for malignant behavior. No more predictive than simple light microscopy, therefore not cast effective.

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NOTES

NEW CONCEPTS IN ANTIMICROBIAL SUSCEPTIBILITY TESTING: THE MUTANT PREVENTION CONCENTRATION AND MUTANT SELECTION WINDOW APPROACH

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Running head: Antimicrobial Susceptibility Testing

Abstract
Current measurements of antimicrobial susceptibility or resistance utilize a standardized bacterial inoculum (10^5 cfu/ml) exposed to varying drug concentrations in a test tube. Following incubation under ideal conditions, the lowest drug concentration inhibiting growth is the minimum inhibitory concentration (MIC). When the MIC exceeds the amount of drug that can be safely achieved in the body, we call these micro-organisms resistant; established breakpoints for various "bug-drug" conditions are used to categorize micro-organisms as susceptible, intermediate or resistant. MIC testing has been used for decades to guide antimicrobial therapy and remains an important measurement for infectious diseases. More recently, the mutant prevention concentration (MPC) has been described as a novel measurement of *in vitro* susceptibility or resistance and is based on the testing of larger bacterial inocula, i.e. $\geq 10^9$ cfu/ml – such as those associated with some infections in humans and animals. MPC defines the lowest drug concentration required to block the growth of the least susceptible cell present in high density bacterial populations. MPC testing applies to micro-organisms considered susceptible to the drug by MIC testing. The mutant selection window (MSW) defines the "danger zone" for therapeutic drug concentrations. Minimizing the length of time the drug concentration remains in the MSW may reduce the likelihood for resistance selection during therapy. The MSW is bordered by the MIC and MPC values and the drug concentration range between the measured MIC and MPC values defines the MSW. MPC values, when considered with drug pharmacology, may allow prediction on the probability of resistance selection when bacteria are exposed to antimicrobial agents during therapy for infectious diseases. In today's environment, resistance prevention should be a goal of antimicrobial therapy.

Introduction
Alexander Fleming – one of our forefathers of antimicrobial agents – had tremendous foresight about the use of these drugs. Since the initial introduction into clinical practice of antimicrobial agents, antimicrobial resistance has been a concern - a concern identified by Fleming himself. Fleming commented in 1945, "…But I would like to sound a note of warning….it is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them and the same thing has occasionally happened in the body." Undoubtedly, Fleming was warning against exposing bacteria to insufficient concentrations of drug and that doing so would ultimately encourage resistance selection.

Today, there is little doubt that we have a global pandemic of antimicrobial-resistant micro-organisms: in human infectious diseases, drug resistance concerns are seen with *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter* species and enteric gram-negative bacilli expressing extended spectrum beta-lactamase (*Escherichia coli*, *Klebsiella* spp. and others)[1]. Some of these same micro-organisms are also a concern in veterinary medicine, in addition to micro-organisms such as *Staphylococcus pseudintermedius* (previously *intermedius*). While regional variations in resistance rates exist for various bacteria/drug combinations, the overall trend has impacted on individual patients, society, the economics of managing infections, and our approach to the empirical use of antimicrobial compounds for both inpatient and outpatient management.

In veterinary medicine, as in human medicine, antimicrobial agents are approved based on the demonstration of non-inferiority of a new drug when compared to a standard antibiotic agent already approved for a specific indication. Clinical trials may fail to take into account various microbiological or pharmacological parameters that could be used to determine optimal versus suboptimal dosing and the potential for resistance selection as prevention. Such parameters may not necessarily affect clinical outcome, but may have a huge impact on the selection of drug resistant pathogens. Clearly, our measurement of antimicrobial susceptibility and the resultant susceptibility or resistance result influences (or should influence) optimization of therapy.

Correlating *in vitro* measurements of antimicrobial susceptibility and the clinical impact

Johnson commented on the predictive value of *in vitro* clinical methods used to evaluate antimicrobial efficacy.[2] In hosts with normal immune defences, a micro-organism susceptible to an antimicrobial agent, as indicated by a low minimum inhibitory concentration (MIC), has an excellent predictive value for a favourable clinical outcome. Antimicrobial resistance as indicated by a high MIC is usually predictive of an unfavourable outcome. Higher MICs usually indicate a greater likelihood of clinical failure. Similar points were made by Johnson regarding hosts with endocarditis, meningitis or deficient immune defences. This, therefore, suggests that the *in vitro* measurement of antimicrobial susceptibility has utility in clinical practice. Unfortunately, MIC testing utilizes a bacterial inoculum that may not be representative of bacterial burdens present during infection (urinary tract, respiratory tract, or central nervous system).[3]

Minimum inhibitory concentration testing

Determination of *in vitro* susceptibility of a pathogen to an antimicrobial agent can be performed by disk diffusion or by the measurement of the MIC, the lowest drug concentration inhibiting or blocking the growth of 10^5 colony forming units/ml (cfu/ml) of the bacterium. Susceptibility testing is controlled for incubation in or on appropriate media, atmosphere, temperature and duration of incubation. Methods for MIC testing include broth microdilution, agar dilution or the E-test. For broth microdilution testing, drug is added to medium in a 96 well microtitre tray and serially diluted to the desired drug concentration range to be tested. Following addition of micro-organism, the assay is incubated for 18-24 hours. The lowest drug concentration preventing visible growth is recorded as the MIC. For agar dilution testing, agar plates incorporating antimicrobial drug at predetermined drug concentrations directly into the medium are inoculated with a known concentration of micro- organisms to the surface of the agar plate. Following incubation, the lowest drug concentration inhibiting growth is the MIC. For E-test, the appropriate inoculum of micro-organism is inoculated over the entire surface of an agar plate and an E-test strip containing gradations of drug concentrations is added to the surface of the plate and incubated. Following incubation, the point on the E-test strip that intersects the line of bacterial inhibition is recorded as the MIC. Antimicrobial susceptibility or resistance is then determined by comparing the measured MIC value to previously established breakpoints that take into account: 1) the drug's *in vitro* activity, 2) achievable and sustainable drug concentrations within the host, 3) distribution and elimination data and 4) drug toxicity. For an MIC recorded at or below the susceptibility breakpoint, the micro-organism is considered susceptible.[4] For MICs recorded above the susceptibility breakpoint, the micro-organism is classified as non-susceptible or resistant. Readers should refer to the relevant Clinical and Laboratory Standard Institute (CLSI) recommended documents (i.e. CLSI document M31-A3) for breakpoints used in veterinary medicine.

In vitro susceptibility testing based on utilization of standardized bacterial inocula (10^5 cfu/ml) has been the foundation of susceptibility testing for decades. Most agree that this form of standardized susceptibility testing has been useful clinically and does serve as a guide for the management of patients with infectious diseases. The patient group that benefits most from susceptibility testing remains debatable: outpatients with self limiting mild to moderate infections, vs inpatients with sepsis. While the correlation between an *in vitro* susceptibility result and clinical outcome is not 100% - other factors play a role in determining outcomes- the test is still useful. In some instances, patients treated with a seemingly appropriate antimicrobial (i.e. one to which the micro-organism is susceptible *in vitro*) may still fail to respond clinically, while those treated with an inappropriate antibiotic (i.e. one to which the micro-organism is resistant) may still show a favourable clinical response. *In vitro* measurements cannot account for the host's immune response which is necessary for successful recovery from infectious diseases. Antimicrobial agents remain an adjunct therapy to the host's natural defences.

New measurements of *in vitro* antimicrobial susceptibility

The mutant prevention concentration (MPC) was described by Dong *et al.*[5] as a novel *in vitro* measurement of antimicrobial susceptibility, and also as the probability of mutant subpopulations being present in high density bacterial populations. Testing fluoroquinolones against *Staphylococcus aureus* and *Mycobacterium smegmatis* strains, these investigators determined that as the number of bacterial cells exposed to drug *in vitro* increased, two distinct regions in the concentration for inhibition of bacterial growth were recognized. The first region was approximated by the MIC drug concentration, at which and at higher drug concentrations, viable micro-organisms could be isolated from drug containing agar plates. Upon molecular analysis, these organisms were found to have mutations conferring reduced susceptibility or resistance to the fluoroquinolone compound being investigated. The second region, the drug concentration that blocked the growth of even these mutant cells, was termed the *mutant prevention concentration* (MPC). The MIC drug concentration is typically lower than the MPC drug concentration, suggesting that prevention of growth of mutant subpopulations from high density bacterial inocula requires higher drug concentrations. This was confirmed in a subsequent report on MPC measurements of fluoroquinolones against clinical isolates of *Streptococcus pneumoniae* [6].

For drugs such as fluoroquinolones where resistance usually arises de novo, the MPC can be defined as the antimicrobial drug concentration that would require a micro-organism to possess two concurrent mutations at two different metabolic steps to grow in the presence of the drug. For fluoroquinolones, one can consider the MPC as the drug concentration required to block the growth of first step resistant mutants. MPC may also be defined as the MIC of the most resistant first step resistant cell present in the population. MPC measurements only apply to micro-organisms deemed to be susceptible to an antimicrobial compound by current recommended susceptibility criteria and breakpoints.

Mutant prevention concentration testing is technically more demanding than MIC testing ($\geq 10^9$ CFUs versus 10^5 cfu/ml respectively). MPC testing requires drug containing plates to be prepared, a centrifugation step may be required, and some organisms (i.e. *Streptococcus pneumoniae*) may be difficult to grow to a density of 10^{10} CFU/ml. Table 1 summarizes some comparative features of MPC testing of various micro-organisms. Briefly, bacterial strains to be tested are subcultured to multiple agar plates (3-8) and incubated for 18-24 hours under ambient conditions for the micro-organism (i.e. O_2 versus 5% CO_2 at 35-37°C). The next day, the complete contents of the inoculated agar plates are removed with a sterile swab and transferred to liquid broth (100-500 ml). The inoculated broth is then incubated for 18-24 hours under

ambient conditions. The next day, the broth culture either contains the necessary bacterial density or it must be centrifuged and the pellet resuspended in a lower volume of fresh broth media. Once the bacterial density is deemed to be correct, $\geq 10^9$ CFUs are inoculated to drug containing agar plates and incubated under ambient conditions. Cultures are read at 24 and 48 hours and the lowest drug concentration preventing growth is the MPC. A schematic diagram showing the method for MPC testing is shown in Figure 1.

Clearly, the current method of MPC testing is labour intensive and does not yet lend itself to easy implementation in clinical laboratories. Hesje and Blondeau[7] compared a modified microbroth dilution method to the agar dilution method for determining MPCs.[7] In this study, gatifloxacin and moxifloxacin were tested against a control strain of *Staphylococcus aureus* (American Type Culture Collection #29213) and against two clinical isolates. For this modified method, 10^1 to 10^9 cfu/ml test micro-organisms were exposed to doubling drug concentrations in wells of microtiter plates. Following incubation under ambient conditions, the MIC was determined from the 10^5 cfu/ml inoculum and the MPC from inocula $\geq 10^7$ cfu/ml. By agar dilution measurements, MPC values for all 3 strains were $\geq$4 μg/ml for these drugs. For inocula of 10^1-10^4 cfu/ml, MIC values ranged from 0.031 to 0.125 μg/ml and at 10^5 cfu/ml the MICs were 0.063-0.125 μg/ml. At 10^7-10^9 cfu/ml, MPC values were $\geq$4 μg/ml. For 10^1-10^4 cfu/ml, MICs to moxifloxacin ranged from 0.016-0.031 μg/ml and all strains had MICs of 0.031 at the 10^5 cfu/ml inoculum. For inocula of 10^7-10^9 cfu/ml, MPC values were $\geq$4 μg/ml. Similar observations have been made with testing of gatifloxacin and moxifloxacin against a control strain and clinical isolates of *S. pneumoniae* (Blondeau, unpublished data). Further validation of this method is ongoing with micro-organisms recovered from human and animal infections and tested against a broader range of antimicrobial agents. One potential additional step required with this new method relates to cellular debris present in wells at the higher bacterial densities. This may make visual interpretation difficult. In such situations, subculturing of the wells to a drug containing agar plate (same drug concentration as that in well of plate) may be necessary to confirm the endpoint.

The potential value of performing susceptibility measurements on higher bacterial populations.

Firsch *et al.*[8] estimated that in patients with pneumococcal pneumonia, the total bacterial burden present during acute infection ranged from 10^{10} to 10^{12} micro-organisms. This single observation alone suggests that many patients may be infected with greater numbers of bacterial organisms than the numbers used in current standardized MIC susceptibility testing. Subsequent to this, Feldman *et al.*[9] reported bacterial counts in cerebral spinal fluid (CSF) ranging from $4.5\text{x}10^3$ to $3\text{x}10^8$ cful/ml and suggested that persistence of a positive culture may be related to an initial high concentration of bacteria. Fagan *et al.*[10] reported *Haemophilus influenzae* and *Streptococcus pneumoniae* bacterial counts of $\geq 10^7$ cful/ml from protected brush specimens from patients with acute bacterial exacerbations of chronic bronchitis. Bingen *et al.*[11] reported CSF bacterial counts ranging from 2x10 to $4\text{x}10^9$ cfu/ml; $\geq 10^7$ cfu/ml for *H. influenzae*, *N.meningitidis*, *S. pneumoniae*, *E. coli* K1 and *S. agalactiae*. Clearly, these reports suggest higher bacterial burdens during infection, prompting one to question if higher bacterial inocula should be used for susceptibility testing. It seems reasonable that higher bacterial burdens are also likely present in infected animals.

Since the description of the MPC concept by Dong *et al.*[5],numerous peer reviewed publications and abstracts have reported MIC and MPC values for various antimicrobial agents against Gram-positive and Gram-negative microorganisms. A summary of MIC and MPC data is presented in Table 2. To date, the clinical significance of MPC measurements has not been fully elucidated; however, reports have been published showing the selection of drug resistant micro-organisms (human pathogen *Streptococcus pneumoniae*) to the treatment drug during therapy.[12, 13]

Figures 2 and 3 show schematically the process of resistance selection when a high density bacterial population is exposed to an antimicrobial agent. It is worth recalling that it only requires one spontaneous mutation to the exposed agent for the culture to become a 10^{12} population following overnight incubation. Figure 2 illustrates how rapidly one or two resistant strains can overcome the initially susceptible population, possibly leading to adverse clinical outcomes.

For patients with normal immunity (depicted in Figure 2 by letter B) both susceptible and resistant cells are cleared. In immunocompromised patients, those with prior infection, those with prior antimicrobial exposure or those patients that appear to be failing therapy for acute infection, it can be argued that continued proliferation of resistant micro-organisms to the point where they breach the immune threshold may result in a bacterial population (depicted by letter A in Figure 2) with a predominance of resistant micro-organisms. Alternatively, clearance and eradication may occur as part of the overall patient response (depicted by C in Figure 2). In bovine respiratory disease, variables such as weather, shipment, co-mingling and other stressors may further compromise the animal and potentially predispose them to prolonging the recovery from infections and thereby, increase the risk for resistance selection. As depicted in Figure 3, dosing based on MPC drug concentrations may reduce the overall bacterial numbers and also prevent the selective amplification of the resistance subpopulation if present as part of the total bacterial burden. MPC dosing may not reduce the likelihood that at risk patients may become infected with a new pathogen.

The "danger zone" for the drug selective amplification of resistant subpopulations is postulated to occur in the mutant selection window (MSW), Figure 4. We previously reported from *in vitro* experiments that drug concentrations exceeding the MPC drug concentration resulted in inhibiting susceptible and mutant micro-organism growth.[3] For drug concentrations falling below the MIC, neither mutant nor susceptible cells are inhibited. For drug concentrations falling within the MSW, susceptible cells are likely inhibited as the drug concentration is in excess of the MIC, however, mutant cells will not be inhibited as the drug concentration is below the MPC. Thus therapeutic drug concentrations that lead to clinical cure may, in fact, be the same drug concentration that selectively amplifies the mutant fraction present in high density bacterial burdens. Dosing to achieve drug concentrations in excess of the MPC likely blocks susceptible and mutant cell growth.

In vitro killing studies have been used to determine if an antimicrobial agent exhibits bacteriostatic versus bactericidal activity and as well, such studies were used to assess the extent and rate of killing of antimicrobial agents. Historically, traditional kill studies were based on bacterial inocula of 10^5 cfu/ml and antibiotic drug concentrations that were multiples if the MIC (i.e. 1x MIC, 2x MIC, 4x MIC, 10x MIC, etc.). We previously argued that as bacterial burdens during infection exceed 10^5 cfu/ml then perhaps kill studies should be based on bacterial densities of 10^6-10^9 cfu/ml. Two such studies have been published to date detailing kill studies using higher bacterial inocula. In those reports with *Streptococcus pneumoniae* and

fluoroquinolones, it was shown that killing of 10^6-10^9 cfu/ml using the measured MIC drug concentration was slow and incomplete and in some instances, micro-organism growth occurred in the presence of the drug concentration tested. Such observations are not completely surprising as the MIC is a measurement of inhibition of growth versus killing, however, $\log_{10}$ reductions are measurable at this drug concentration, indicating killing occurs. When killing of 10^6-10^9 cfu/ml was attempted with MPC drug concentrations, killing was more rapid and complete suggesting MPC drug concentrations were necessary to effect >99% reduction in high density bacterial populations.

Blondeau *et al.*[14] compared the killing of bovine isolates of *Mannheimia haemolytica* by enrofloxacin, florfenicol, tilmicosin and tulathromycin using the measured MIC and MPC drug values. In these experiments, micro-organisms were grown to densities of 10^9 cfu/ml and then diluted to give densities ranging from 10^6-10^9 cfu/ml. Bacterial cultures were exposed to either the MIC or MPC drug concentrations, aliquots were sampled in triplicate at 0, 30 minutes, 1, 2, 3, 4, 12 and 24 hours, plated, incubated under ambient conditions and the reductions in viable micro-organisms recorded. In these experiments, the MIC values (μg/ml) for enrofloxacin, florfenicol, tilmicosin and tulathromycin were 0.16, 0.25-2, 0.5-4 and 0.2-2 respectively; MPC values (μg/ml) were 0.125-0.5, 2-8, 4-64 and 2-4 respectively. Exposure of 10^6-10^9 cfu/ml to the MIC drug concentrations gave a growth to 2.4 $\log_{10}$ reduction in viable cells by 4 hours for florfenicol compared to growth to 0.13 $\log_{10}$ reduction, growth to 0.57 $\log_{10}$ reduction, growth to 0.35 $\log_{10}$ reduction for enrofloxacin, tilmicosin and tulathromycin respectively. All drugs yielded a growth to 1.37 $\log_{10}$ reduction of the 10^9 cfu/ml inocula by 24 hours. Exposure of 10^6-10^9 cfu/ml of micro-organism to MPC drug concentrations gave a growth to 3 $\log_{10}$ reduction, growth to 0.49 $\log_{10}$ reduction and growth to 0.1 $\log_{10}$ reduction in viable cells by 30 minutes to 1 hour for enrofloxacin, florfenicol, tilmicosin and tulathromycin respectively. A growth to 6 $\log_{10}$ reduction was seen to the four drugs by 12 to 24 hours with enrofloxacin showing the greatest reductions followed by florfenicol, tilmicosin and tulathromycin. It was concluded from this study that killing of the *Mannheimia haemolytica* strains was less efficient at the MIC drug concentrations but was more complete and efficient at MPC drug concentrations as described above. Dosing to achieve MPC minimizes resistance selection and ensures more efficient and rapid killing.

In a follow up study, the same authors examined the concentration dependent killing of *M. haemolytica* isolates by enrofloxacin and in addition to conducting kill studies based on the MIC and MPC drug concentration values, drug concentration values representing the maximum serum and maximum tissue drug concentrations were also used in kill assays involving 10^6-10^9 cfu/ml.[15] When *M. haemolytica* was exposed to enrofloxacin at the maximum serum drug concentration, a 1.7-2.4 $\log_{10}$ reduction (96-99% killing) was seen at 1 hour. Similar values were also seen following exposure to the maximum tissue drug concentration. This study suggests that for concentration dependent antibiotics, dosing to achieve drug concentrations at or above the MPC drug concentration is necessary to effect a substantial reduction in viable micro-organisms – especially high bacterial burdens such as those seen during infection.

Hansen *et al.*[16] suggested that the period during which drug concentrations remain in excess of the MPC may be important for restricting mutant growth. In studies published with *Streptococcus pneumoniae*, a >99% reduction in viable cells occurred between 6-12 hours of exposure to various fluoroquinolones when micro-organisms were exposed to the MPC drug concentration in time kill experiments.[17, 18] Investigations with macrolide compounds showed similar results, i.e. that a minimum amount of time at or above the measured MPC value was

necessary to effect substantial reduction in viable micro-organisms.[19] Killing high density bacterial populations (10^6-10^9 cfu/ml) with MIC drug concentration is slow and incomplete. Such data might suggest that time above the MSW of at least 6 hours may be important for ensuring substantial reductions of high density bacterial inocula as the kill experiments highlighted above were performed using bacterial inocula ranging from 10^6-10^9 cfu/ml – inocula consistent with the MPC approach.

In summary, MPC testing is a unique approach to *in vitro* susceptibility testing as it utilizes bacterial inocula which better reflect bacterial burdens present in a number of infections. Such testing might provide greater insight into the true dynamics of these high density bacterial populations when exposed to certain antimicrobial compounds.

Smith *et al.*[20] suggested that the MPC method of testing only applies to fluoroquinolone compounds. Subsequently, numerous studies have elucidated MPCs against a wide variety of antimicrobial compounds and bacterial pathogens. Molecular explanations of elevated MPC values remain unresolved for many "bug-drug" combinations. A number of these observations are summarized in Table 2. The *resistance prevention concentration* was coined as an all encompassing terminology to define the antimicrobial drug concentration that blocked the growth of the least susceptible micro-organims present in high density bacterial inocula and was independent of the mechanism of resistance of those mutant cells.[3] In fact, MPC and RPC testing is synonymous and it remains important to remember that MPC defines the mutant prevention concentration and not the *mutation* prevention concentration. The measurement of MPC is to determine the drug concentration necessary to block the growth of the least susceptible cell in the population and is independent of the mechanism of resistance.

Not all mechanism of antimicrobial resistance arise de novo. In many instances, resistance may be the result of horizontal gene transfer by an acquired genetic element (plasmid, transposon) containing resistance-conferring genes. Such examples include beta-lactamase resistance on plasmids and tetracycline resistance on transposons. MPC measurements in these instances are unlikely to apply as micro-organisms harbouring resistance conferring genes already demonstrate elevated MICs and are resistant by CLSI criteria. MPC testing is only relevant against bacterial strains susceptible to the drug by CLSI criteria. Once the organism is considered resistant by MIC testing, MPC measurements are not useful. While for some "bug-drug" combinations the major mechanism of resistance is by the acquisition of a resistance gene, this does not exclude the potential for other mechanisms of resistance that are potentially preventable by MPC testing of susceptible strains.

Observations for fluoroquinolones in dermatology

For many antimicrobial agents, information regarding achievable or sustainable drug concentrations in the skin are somewhat elusive. For two fluoroquinolones – enrofloxacin and marbofloxacin- skin drug concentrations are available. For enrofloxacin, skin drug concentrations range from 1.7-1.9ug/ml in dogs and cats and in inflamed skin in the dog, drug concentrations are 3.1ug/ml after 3 days of therapy. As such, for *S. intermedius*, an organism with an MIC of 0.063ug/ml would yield a maximum tissue to MIC ratio between 26.9-30.1. As indicated in Table 2, the MPC for enrofloxacin against *S. intermedius* was 0.5ug/ml which would yield a tissue max/MPC ratio of 3.4-6.2.

For marbofloxacin, skin drug concentrations in dogs were 3.2ug/ml and the tissue max/MIC ratio would be 6.4 (at an MIC of 0.5ug/ml; Table 2). For an MPC of 1 ug/ml (Table 2), the tissue max/MPC ratio would be 3.2.

For both fluoroquinolones above, it is clear that higher or lower MIC and/or MPC values would influence the magnitude of the tissue max/MIC or MPC ratios. The exact clinical impact of these observations has not yet been studied. Regarding resistance prevention, higher ratios are likely to be more effective, however, this has not been evaluated clinically.

MIC and MPC measurements need to be considered along with PK/PD data.

The action of antimicrobial agents are clearly affected by pharmacokinetic (PK) and pharmacodynamics (PD) parameters, with PK defining the fate of the drug in the body (eg. absorption, transformation, distribution, elimination) and PD defining the effect of the drug on the body and infecting organisms (including the drug's mechanism of action and efficacy).[21]

PK/PD principles have been used to characterize various compounds based on the mechanism by which they exert their antibacterial activity. Figure 5 shows the schematic representation of a drug curve. Three PK/PD relationships have been established and applied to various classes (and within classes to specific agents/species) of antimicrobial compounds. For *concentration dependent* antimicrobial compounds, the C_{max} to MIC ratio as well as the area under the curve (AUC) to MIC ratio have been shown to be important predictors of outcome following antimicrobial therapy. Antimicrobial compounds that are considered *time dependent* agents exert their antibacterial activity based on the time the drug concentration remains in excess of the MIC. A C_{max} to MIC ratio of >8-12 is felt to be important for positively impacting clinical outcome and reducing the likelihood for resistance selection. For agents characterized based on a AUC/MIC ratio, a ratio of >125 has been suggested as being necessary for Gram-negative micro-organisms and 30-50 for Gram-positive pathogens. The absolute values of these ratios have been debated and have yet to be completely resolved.[22-24] Table 3 lists antimicrobial agents used in human and veterinary medicine and summarizse the PK/PD parameters that characterize their mechanism of antimicrobial action.[25]

It is clear that in patients being treated with antimicrobials, a number of possible scenarios may occur [26]:

1. Clinical resolution with complete eradication of the pathogen from the infected site.
2. Clinical resolution with persistence of the micro-organism in the host.
3. Clinical resolution with persistence of the pathogen that now is resistant to the treatment antimicrobial.
4. Clinical failure with micro-organism proliferation.
5. Clinical failure with proliferation of an antimicrobial resistant pathogen.
6. Clinical failure due to infection with a secondary pathogen.

Optimal antimicrobial therapy would be that which results in a favourable clinical outcome where the infected micro-organism has been eliminated and resistance selection prevented. Does it seem likely that a different AUC/MPC value would apply for resistance prevention against a Gram-positive versus a Gram-negative organism? Unfortunately, such data is not readily available. Zinner *et al.*[27] used an *in vitro* pharmacodynamic model to test moxifloxacin against *S. pneumoniae* isolates and suggested that an AUC_{24}/MIC >100h may protect against selection of resistant *S. pneumoniae* mutants. Unfortunately, this value does not tell us what the AUC/MPC value might have been. Metzler *et al.*[28] determined MIC and MPC values for meticillin-

susceptible strains of *Staphylococcus aureus* tested against gatifloxacin, gemifloxacin, levofloxacin and moxifloxacin. $AUC_{0\text{-}24}/MPC_{90}$ ratios were calculated for total and free drug and were as follows respectively: 51.3/41, 16.8/6.7, 48/35.5 and 190/119.7. Unfortunately, it is uncertain as to what these values actually mean given that specific studies to investigate what the AUC/MIC values for *S. aureus* and fluoroquinolones need to be, has yet to be determined. Similarly, Blondeau *et al.*[3] calculated $AUC_{0\text{-}24}/MPC_{90}$ ratios for the same four fluoroquinolones against clinical isolates of *Streptococcus pneumoniae.* Those values respectively were 26.7, 18.4, 12 and 47.5: free drug values respectively would be 21.4, 7.4, 8.8 and 29.9. Once again, it remains unclear as to the significance of these values given that the appropriate studies have yet to be completed to determine what the AUC/MPC values need to be.

At least one study has compared MIC and MPC values for bovine isolates of *M. haemolytica* against enrofloxacin, florfenicol, tilmicosin and tulathromycin. In this report, MIC_{90} values (μg/ml) were 0.125, 0.5, 8 and 1 respectively; MPC_{90} values (μg/ml) were 0.5, 4, $\geq$32 and 8 respectively. Similar calculations have now been completed for enrofloxacin and *M. haemolytica*; AUC/MIC and AUC/MPC ratios were reported to be 160 and 80 respectively.[29] as florfenicol, tilmicosin and tulathromycin are classified as time dependent antimicrobials, $T>MIC_{90}$ and $T>MPC_{90}$ for these 3 agents were as follows respectively: 64 and ~3 hours; >24-<48 and 0 hours; $\geq$172 and 0 hours.

Olofsson *et al.*[30] investigated the selection of ciprofloxacin resistance in *Escherichia coli* in an *in vitro* kinetic model to determine the relationship between drug exposure and mutant prevention concentration. Two ciprofloxacin susceptible strains and 1 strain containing a first-step gyrase mutation were evaluated. The parameters investigated included time>MPC (T>MPC), C_{max} and AUC/MPC. From their investigations, the authors concluded that neither of T>MPC nor C_{max} proved to be single correlates for preventing resistance development in their experiments and against the strains tested. Against the two wild type susceptible strains, the authors found that an AUC/MPC ratio of $\geq$22 was a single pharmacodynamic index that predicted prevention of resistant mutant development. The authors also concluded that further studies are warranted to verify the usefulness of this pharmacodynamic index for the design of dosing regimens. As yet, similar types of experiments have not been published related to Gram-positive pathogens, therefore, it remains unclear if a different AUC/MPC value would be necessary for Gram-positives versus Gram-negatives as has been argued for AUC/MIC values – particularly with *S. pneumoniae*. More recently, measurements of MPC have been completed with various veterinary pathogens and antimicrobial agents. A summary of these values are presented in Table 2.

As previously stated, optimal antimicrobial therapy would be that which results in a favourable clinical outcome, eradicates the infecting pathogen while minimizing likelihood for resistance selection during drug exposure. However, such a strategy may be prevented by adverse events observed at these higher, but microbiologically necessary drug concentrations. Additionally, higher dosages needed to prevent resistance development may cost more. Unfortunately use of agents which fail to cure without resistance selection are likely to drive costs higher overall as clinicians have to use more expensive agents to overcome resistant pathogens. Treatment

guidelines for a variety of infectious diseases in humans are beginning to address a new approach to prescribing antibiotics based on a century old concept; that of hitting hard and hitting fast, however, formalized guidelines have not materialized in veterinary medicine.[31] Using the highest, safest antibiotic drug concentrations may provide excellent clinical outcomes with minimal side effects while preserving the drug class for future patients. Application of PK/PD to MPC concepts to avoid selecting mutant strains within bacterial populations can help improve both short term and long term outcomes.

In summary, MIC testing remains a useful guide for determining an organism's susceptibility to antimicrobial agents and has been the cornerstone of susceptibility testing for decades. Unfortunately, MIC testing may not fully take into account the true dynamics of higher density bacterial populations such as those associated with infection. MIC testing at an inoculum of 10^5 CFU/ml does not allow for the detection of resistant sub-populations that may arise in bacterial populations 10^6 to 10^8 or lower. As such, MPC testing may offer some value for guiding optimal antimicrobial therapy as it provides practical information on drug concentrations necessary to restrict mutant growth during infections where high bacterial burdens are likely present. Restricting mutant growth is desirable – especially given increasing antimicrobial resistance trends.

Measurements of MPC are often done using a single drug tested against one strain of bacteria. In some instances, single drug therapy may be insufficient and a combination of drugs may be warranted. Combination drug therapy was previously measured by us with clinical isolates of *Pseudomonas aeruginosa*. MPC values for either drug alone were outside of clinically achievable drug concentrations but combination MPC values were within achievable drug concentrations. This observation may have important implications for therapy against more difficult to treat pathogens or against organisms where multiple resistance mechanisms are common.

Sir Alexander Fleming wrote in 1946 "…the greatest possibility of evil in medication is the use of too small doses so that instead of clearing up infection the microbes are educated to resist penicillin and a host of penicillin-fast organisms is bred out, which can be passed to other individuals and from them to others, until they reach someone who gets septicemia or pneumonia which penicillin cannot save." Clearly, many unanswered questions exist regarding the MPC and MSW concepts. Further studies will be necessary to refine our understanding of the MSW and strategies to narrow or close the window so as to minimize the amount of time that drug concentrations remain in the "danger zone".

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Table 1: Comparison of protocols for MPC testing of various microorganisms.[a]

Microorganism	# starter plates inoculated	Duration of incubation	Atmosphere	Subculture to liquid media[b]	Duration of incubation	Centrifugation required[c]	Incubation duration to define endpoint
S. pneumoniae	6	18-24 hrs	5% CO_2	Yes 500 ml THB	18-24 hrs	Yes	24-48 hrs
S. aureus	3	18-24 hrs	O_2	Yes 100 ml MHB	18-24 hrs	No	24-48 hrs
E. coli	2-3	18-24 hrs	O_2	Yes 100 ml MHB	18-24 hrs	No	24-48 hrs
P. aeruginosa	2-3	18-24 hrs	O_2	Yes 100 ml MHB	18-24 hrs	No	24-48 hrs
Klebsiella spp.	2-3	18-24 hrs	O_2	Yes 100 ml MHB	18-24 hrs	No	24-48 hrs
Citrobacter spp.	2-3	18-24 hrs	O_2	Yes 100 ml MHB	18-24 hrs	No	24-48 hrs
S. intermedius	2-3	18-24 hrs	O_2	Yes 100 ml MHB	18-24 hrs	No	24-48 hrs
M. haemolytica	4-5	18-24 hrs	O_2	Yes 100 ml HTM	18-24 hrs	Yes	24-48 hrs
P. multocida	3-4	18-24 hrs	O_2	Yes 100 ml MHB	18-24 hrs	No	24-48 hrs
H. influenzae	7-8	18-24 hrs	CO_2	Yes 100 ml HTM	18-24 hrs	Yes	24-48 hrs

[a]Please see text for description.
[b]THB=Todd-Hewitt broth; MHB=Mueller-Hinton broth.
[c]5000 rpm for 10 minutes at 4°C.

Table 2: Summary of MIC and MPC data. Modified from Hesje[26].

Organism	Antimicrobial Agent	n	MIC_{90}*	MPC_{90}*	Ref
Streptococcus pneumoniae					
	Moxifloxacin	99	ND	1‡	6
	Trovafloxacin	99	ND	2‡	6
	Gatifloxacin	100	ND	2‡	6
	Grepafloxacin	95	ND	4‡	6
	Levofloxacin	101	ND	4‡	32
	Ciprofloxacin	38	2	8	32
	Ofloxacin	38	2	8	32
	Azithromycin	177	0.125	4	33
	Clarithromycin	206	0.063	1	33
	Erythromycin	201	0.125	2	33
Pen Susceptible	Moxifloxacin§	21	2	4	34
	Gatifloxacin§	21	4	8	34
	Gemifloxacin§	21	0.25	2	34
	Levofloxacin§	21	8	≥16	34
	Azithromycin	49	0.5	≥8	35
	Clarithromycin	49	≥1	≥4	35
	Erythromycin	49	≥8	≥4	35
	Garenoxacin	427	0.125	0.5	36
Pen Intermediate	Moxifloxacin	7	2	≥8	34
	Gatifloxacin	7	4	≥16	34
	Gemifloxacin	7	1	2	34
	Levofloxacin	7	8	≥32	34
	Azithromycin	10	>16	≥8	35
	Clarithromycin	10	≥1	≥4	35
	Erythromycin	10	≥8	≥4	35
	Garenoxacin	80	0.125	0.5	36
Pen Resistant	Gemifloxacin	8	0.063	0.5	37
	Levofloxacin	8	1	8	37
	Garenoxacin	17	0.063	0.5	36
Staphylococcus aureus					
MSSA	Ciprofloxacin	4	0.5	2	32
		1	0.125	2	38
	Gatifloxacin	4	0.063	0.063	32
		122	0.25	1	8
		1	0.016	0.125	38
	Ofloxacin	4	0.25	2	32
	Gemifloxacin	1	0.031	0.063	38
		122	0.063	0.5	8
	Levofloxacin	1	0.,125	1	38
		122	0.25	1	8
	Moxifloxacin	1	0.015	0.25	38

		122	0.063	0.25	[8]
	Cefazolin	26	2	64	[39]
	Cloxacillin	26	0.25	2	[39]
	Vancomycin	26	1	4	[39]
MRSA	Ciprofloxacin	1	0.125	1	[38]
	Gatifloxacin	1	0.063	0.125	[38]
		22	8	32	[38]
	Gemifloxacin	1	0.031	0.063	[38]
		22	8	256	[28]
	Levofloxacin	1	0.125	0.5	[38]
		22	>16	128	[28]
	Moxifloxacin	1	0.063	0.125	[38]
		22	4	16	[28]
	Cefazolin	24	16	512	[39]
	Cloxacillin	24	32	>512	[39]
	Vancomycin	24	1	8	[39]
ATCC 6538	Pradofloxacin		0.03-0.06	0.5-0.6	[40]
	Danofloxacin		0.125-0.25	10-11	[40]
	Difloxacin		0.125	16-18	[40]
	Enrofloxacin		0.06-0.125	3-3.5	[40]
	Marbofloxacin		0.25-0.5	3-3.5	[40]
	Orbifloxacin		0.5	8-9	[40]
	sarafloxacin		0.125-0.25	8-9	[40]
	Ciprofloxacin		0.25-0.5	6	[40]
	Moxifloxacin		0.03-0.06	0.8-1	[40]
DSM 11823	Pradofloxacin		0.06	0.2-0.25	[40]
	Enrofloxacin		0.125-0.25	1	[40]
Staphylococcus intermedius ATCC 29663	Pradofloxacin		0.03	0.15	[40]
	Enrofloxacin		0.06-0.125	2-2.5	[40]
	Ciprofloxacin	1	0.03	0.25	A
	Enrofloxacin	1	0.03	0.5	A
	Amikacin		2	≥32	A
	Ampicillin		0.25	≥128	A
	Cefazolin		0.063	16	A
	Doxycycline		2	≥64	A
	Enrofloxacin		0.063	1	A
	Erythromycin		0.5	≥8	A
	Gentamicin		0.25	4	A
	Marbofloxacin		0.5	1	A
	Pradofloxacin		0.063	0.125	A
	Ceftriaxone		1	8	A
	cefofaxime		2	4	A
Escherichia coli	Ciprofloxacin	20	≤0.06	0.5	[41]
	Levofloxacin	20	≤0.06	1	[41]
	Garenoxacin	20	≤0.06	1	[41]
	Moxifloxacin	23	0.031	1	[42]
	Tigecycline	26	0.063	1	

	Amikacin		4	≥32	A
	Ampicillin		8	≥128	A
	Cefazolin		4	128	A
	Ceftriaxone		0.125	16	A
	Cefofaxime		0.125	16	A
	Doxycycline		1	≥64	A
	Enrofloxacin		0.016	0.25	A
	Gentamicin		1	≥8	A
	Marbofloxacin		0.016	0.5	A
	Pradofloxacin		0.016	0.125	A
ATCC 8739	Pradofloxacin		0.015-0.03	0.2-0.25	[40]
	Danofloxacin		0.06	0.5-0.55	[40]
	Difloxacin		0.125-0.25	1.5-1.6	[40]
	Enrofloxacin		0.03-0.06	0.3-0.35	[40]
	Marbofloxacin		0.03	0.25-0.3	[40]
	Orbifloxacin		0.125	1-1.25	[40]
	Sarafloxacin		0.03-0.06	0.5-0.6	[40]
	Ciprofloxacin		0.015-0.03	0.1-0.15	[40]
	Moxifloxacin		0.06-0.125	0.5-0.6	[40]
ATCC 2592	Pradofloxacin		0.008-0.015	0.075-1	[40]
	Enrofloxacin		0.015-0.03	0.15-0.175	[40]
	Marbofloxacin		0.015-0.03	0.2-0.25	[40]
	Ciprofloxacin		0.008-0.015	0.1-0.15	[40]
DMS 10650	Pradofloxacin		≤0.008	0.075-0.1	[40]
	Enrofloxacin		≤0.008	0.125-0.15	[40]
	Marbofloxacin		0.008-0.015	0.175-0.2	[40]
	Ciprofloxacin		≤0.008	ND	[40]
Wild-type	Pradofloxacin		0.015-0.03	0.125-0.15	[40]
	Enrofloxacin		0.03-0.06	0.4-0.5	[40]
	Marbofloxacin		0.03	0.5	[40]
	Ciprofloxacin		0.015-0.03	0.3	[40]
Haemophilus influenzae	Ciprofloxacin	31	0.016	0.5	[43]
	Ofloxacin	31	0.031	0.5	[43]
	Levofloxacin	31	0.016	0.125	[43]
	Moxifloxacin	40	0.031	0.25	[44]
	Gatifloxacin	31	0.031	0.125	[43]
	Gemifloxacin	40	0.008	0.125	[44]
	Azithromycin	40	2	32	[44]
	Telithromycin	40	2	16	[44]
	Clarithromycin	40	8	≥64	[44]
	Cefuroxime	40	16	≥16	[44]
	Ciprofloxacin	26	0.016	0.5	[45]
	Ofloxacin	26	0.031	0.5	[45]
	Moxifloxacin	26	0.031	0.5	[45]
	Gatifloxacin	26	0.016	0.5	[45]
Citrobacter freundii	Ciprofloxacin	20	0.125	2	[41]
	Levofloxacin	20	0.5	2	[41]
	Garenoxacin	20	4	8	[41]

Enterobacter cloacae	Ciprofloxacin	20	≤0.06	1	[41]
	Levofloxacin	20	0.125	4	[41]
	Garenoxacin	20	1	>8	[41]
Klebsiella pneumoniae	Ciprofloxacin	20	≤0.06	1	[41]
	Levofloxacin	20	1	2	[41]
	Garenoxacin	20	0.25	4	[41]
	Moxifloxacin	18	0.25	≥2	[42]
	Ciprofloxacin	20	0.06	1	
	Levofloxacin	20	1	2	
	Garenoxacin	20	0.25	4	
	moxifloxacin	18	0.25	≥2	
Klebsiella oxytoca	Moxifloxacin	6	0.063	0.5	[42]
***Klebsiellsa* spp.**	Tigecycline	24	0.5	8	[46]
Pseudomonas aeruginosa	Ciprofloxacin	20	1	4	[41]
	Levofloxacin	20	4	16	[41]
	Garenoxacin	20	4	≥32	[41]
	Ofloxacin	22	8	16	[43]
	Gatifloxacin	22	4	8	[43]
	Ciprofloxacin	151		4	[47]
	Levofloxacin	151		16	[47]
Mannheimia haemolytica	Enrofloxacin	139	0.125	0.5	[29]
	Florfenicol	135	2	4	[29]
	Tilmicosin	143	8	≥32	[29]
	Tulathromycin	139	1	8	[29]
Salmonella typhimurium	ciprofloxacin	1	0.03	0.5	48
	enrofloxacin	1	0.03	8	48

*Individual MIC or MPC values where only one organism was reported.

‡Adjusted from two-fold overestimation in original publication.

§Against organisms with elevated MICs to levofloxacin of ≥2 mg/L.

MIC=Minimum inhibitory concentration; MPC=Mutant prevention concentration; MRSA=Methicillin-resistant *S. aureus;* MSSA=Methicillin-susceptible *S. aureus*; ND=not determined.
A=Blondeau unpublished.

Table 3: Antimicrobial agents and PK/PD characteristics [25].

C_{max}/MIC	AUC/MIC	T>MIC
Streptomycin	Streptomycin	Benzylpenicillin
Gentamicin	Gentamicin	Amoxicillin
Tobramycin	Amikacin	Cloxacillin
Amikacin	Tobramycin	Carbenicillin
Danofloxacin	Danofloxacin	Cefalexin
Enrofloxacin	Enrofloxacin	Ceftiofur
Marbofloxacin	Marbofloxacin	Cephapirin
Difloxacin	Difloxacin	Florfenicol
Sarafloxacin	Sarafloxacin	Chloramphenicol
Metronidazole	Metronidazole	Erythromycin
	Colistin	Tilmicosin
	Oxytetracycline	Tulathromycin
	Chlortetracycline	Aivlosin
	Doxycycline	Clindamycin
	Azithromycin	Sulfadiazinesulfadoxime
	Clarithromycin	Trimethoprim
	Vancomycin	

Cmax/MIC: maximum serum drug concentration to MIC ratio
AUC/MIC: area under the drug concentration curve to MIC ratio
T>MIC: time serum drug concentrations exceed the MIC over the dose

Figure 1: Schematic Method of MPC Testing

- Basic method:
  - Inoculate plates and incubate
  - Transfer to fresh media
  - Centrifuge and resuspend in fresh media
  - Inoculate drug containing plates with 10^{10} organisms
- Varies by organism

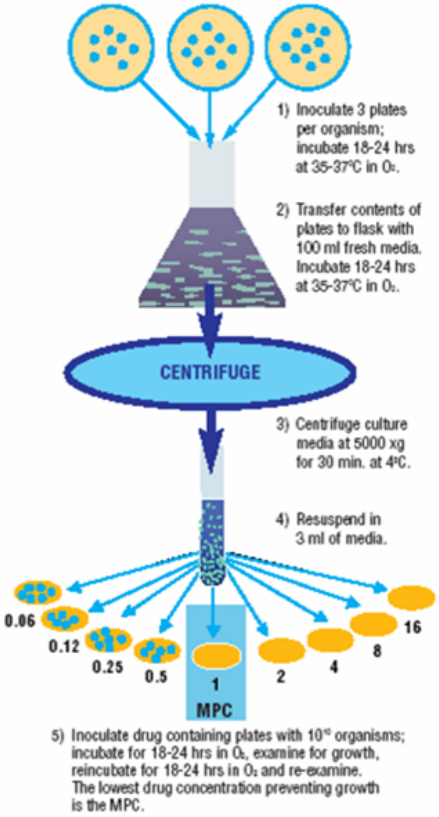
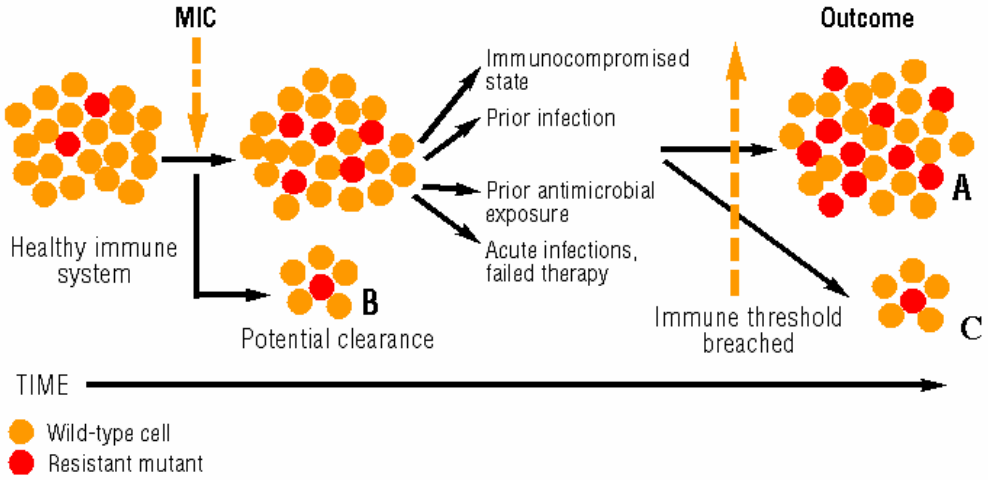


Figure 2: Schematic representation of resistance selection based on MIC drug concentrations.

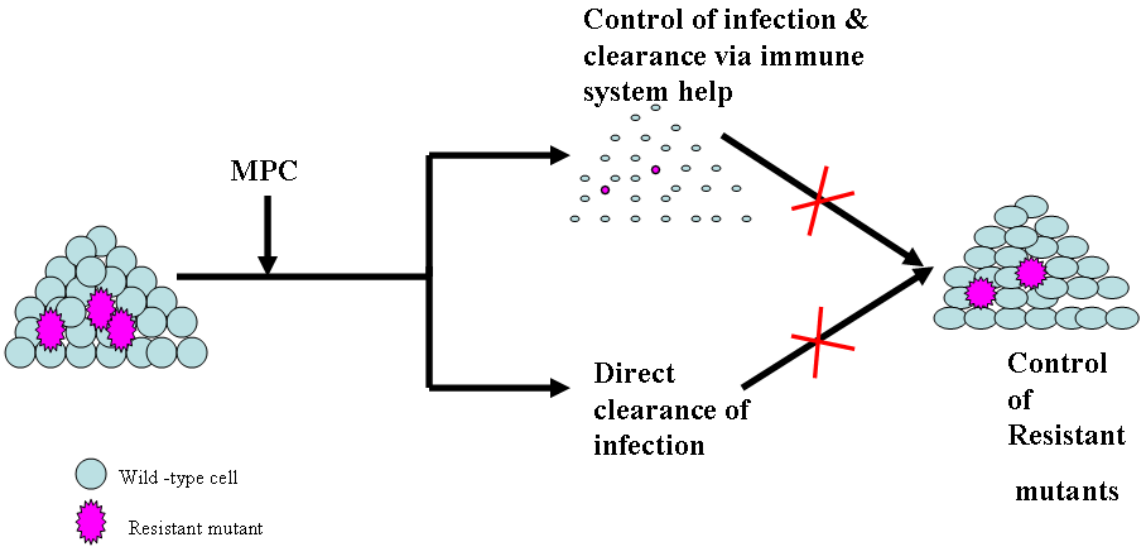


A. Bacterial numbers may not be reduced during clinical failure and/or selection of resistant organisms.

B. Bacterial numbers are reduced during a favorable clinical response and where drug concentrations are sufficient to block mutant growth and/or due to a functioning immune response.

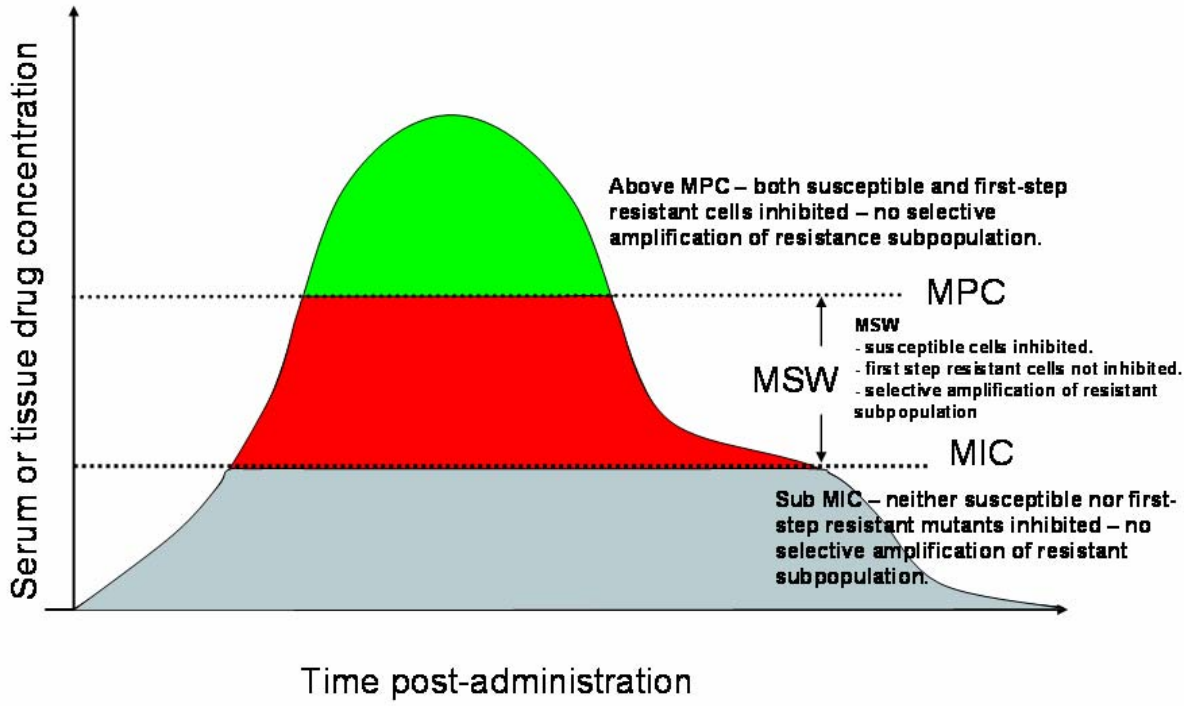
C. Resistant organisms selected during drug therapy and colonizing body surfaces may be reduced over time due to normal competitive microorganisms.

Figure 3: Selective Amplification of resistant mutants.



During infection, both susceptible cells and first-step resistant mutants are present. Dosing at the level of the MPC would result in either direct clearance of infection via lytic drug action, or control of the infection facilitation clearance via the immune system.

Figure 4: Mutant Selection Window (MSW)

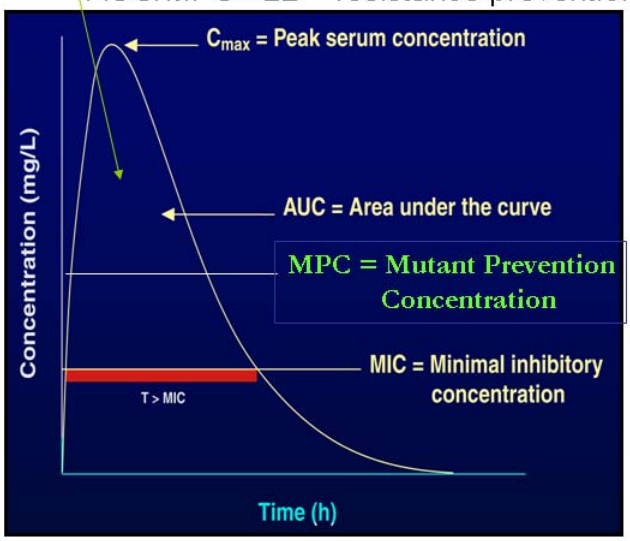


Reproduced with permission from Reference 3.

Figure 5: PK/PD Relationships: Surrogate Markers

- Concentration-dependent
  - Peak/MIC > 8 - 12
  - AUC/MIC = AUIC
    - > 125 Gram –
    - ~30-50 Gram +
  - AUC/MPC = ?
- Time-dependent
  - T > MIC; 40-50%

Olofsson et al, 2006
AUC/MPC >22 = resistance prevention



Cmax/MIC: maximum serum drug concentration to MIC ratio

AUC/MIC: area under the drug concentration curve to MIC ratio

T>MIC: time serum drug concentrations exceed the MIC over the dose

NOTES