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DESIGNING A NEW RANGE OF TOPICAL PRODUCTS: THE ALLERMYL® STORY

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"History is a relentless master. It has no present, only the past rushing into the future"
John Fitzgerald Kennedy

Atopic dermatitis (AD) is possibly the most common cause of pruritus in dogs, affecting as much as 10% of the canine population (Carlotti and Costargent, 1994; Scott et al., 2001).

The disease currently is defined as a genetically-predisposed inflammatory and pruritic allergic skin disease, often associated with IgE antibodies to environmental allergens (Olivry et al., 2001). Much of the therapeutic research consequently has focused on providing solutions to the inflammatory component of the disease, either by the systemic or topical route (corticosteroids, cyclosporine, anti-histamines...), and epidermal changes associated with the inflammatory dermatosis have long been considered downstream participants in the disease pathogenesis (**inside-outside concept**).

However, recent advances in the human field suggest that the epidermal abnormality is not just a secondary phenomenon, but rather a critical, if not the primary, exacerbant of inflammatory skin disease (Elias and Feingold, 2001). It is now recognised that the *stratum corneum* (SC) is not an inert end product but rather a sophisticated biosensor essential in bodily protection at the interface with the hostile terrestrial environment. The SC responds to external perturbations, such as trauma or altered humidity, with a variety of signals that result in stimulation of metabolic responses in the underlying epidermis aimed at normalising SC function. Some of these signals (cytokines, growth factors and a variety of lipid mediators) stimulate skin cascades that initiate not only homeostatic responses, but also inflammatory events in deeper skin layers (**outside-inside paradigm**, Elias et al., 1999). Indeed, several studies in man have evidenced the altered epidermal barrier in patients with atopic dermatitis (Fartasch and Diepgen, 1992; Fartasch, 1994).

Recognising these advances in the knowledge of the aetiology and pathogenesis of inflammatory dermatoses in man, Virbac has tested the relevance of these concepts in veterinary medicine and developed a therapeutic program to address the specific problems of atopic dermatitis in dogs.

1 Selection of active ingredients

1.1 What goes wrong in canine atopic skin ?

Three main features are identified as playing a pivotal role in the disease expression in dogs (Fig. 1):

- Impaired epidermal barrier function, resulting from structural defects.
- Surface microbial overgrowth, acting as aggravating factor.
- Epidermis-initiated immunological events, that lead to inflammation and pruritus.

Strong evidence supports the hypothesis of an allergen challenge occurring in the superficial epidermis in dogs (Olivry and Hill, 2001a). Percutaneous penetration of allergens would be increased in the case of atopic dermatitis, because of defects of the skin barrier at the level of the *stratum corneum* (Koch, 2000). The functional integrity of the epidermis relies on the intercellular lipids surrounding the keratinocytes. These lipids are critical for normal barrier function, maintaining cellular cohesion (Olivry and Hill, 2001b). Among these lipids, linoleic acid, a component of the ceramides, plays a fundamental role (Kwochka, 1993).

As a result of defective epidermal barrier function increased antigenic stimulation of the immune system occurs, together with increased adhesion to corneocytes of surface bacteria and yeast (Scott et al., 2001) and, possibly, excessive transepidermal water loss resulting in dry skin.

These changes provide a medium favourable to the growth of microbial elements present in the natural skin flora of the dog, namely *Staphylococcus intermedius* and *Malassezia pachydermatis*. Microbial proliferation often results and causes further damage to the skin barrier through infection and allergenic stimulation (DeBoer and Marsella, 2001).

Allergens that penetrate into the epidermis are trapped and processed by antigen presenting cells of the immune system (Langerhans cells). Keratinocytes, by far the most numerous cell population in the epidermis, are also now increasingly recognised for their contribution to initiation of local immune reactions, beyond their mere structural function. Proper stimulation (e.g. staphylococcal superantigens) may activate keratinocytes which respond by producing pro-inflammatory cytokines (such as TNF-α) and express leucocyte adhesion molecules on their cell surfaces (Palacio et al., 1997). In the presence of superantigens, keratinocytes thus behave as antigen presenting cells and directly activate T-cells (Scott et al., 2001). *In vivo* overexpression of TNF-α mRNA has been demonstrated in the lesional skin of atopic dogs, as compared to healthy controls (Nuttall et al., 2002).

1.2 Allermyl® triple action response

Development of Allermyl® products was driven by the lessons learnt from new knowledge on the pathogenesis of atopic dermatitis in dogs. An exclusive combination of original molecules was thus designed providing high specific activity to:

- restore cutaneous integrity,
- control aggravating microbial proliferation,
- limit immune and inflammatory reactions.

Linoleic acid (LA) and **gamma-linolenic acid** (GLA) are essential fatty acids of the ω-6 family. LA is incorporated into the intercellular lipid cement of the *stratum corneum* after cutaneous application in the dog (Campbell and Kirkwood, 1993). GLA is a major structural

component of cell membrane phospholipids. Maintenance of cell membrane fluidity is a prerequisite to cellular integrity, in particular for keratinocytes. Topical use of fatty acids with Allermyl® is therefore aimed at restoring epidermal barrier function and thus reducing allergen penetration through the skin.

Piroctone olamine (PO), also called Octopirox, is an antifungal of the hydroxy-pyridone family unrelated to other antiseptics used in veterinary medicine. Members of the "pirox" family are currently used in the human field as topicals to cure onychomycosis and *Malassezia*-related skin disorders. PO has broad *in vitro* activity against major dermal veterinary pathogens, including dermatophytes and yeasts as well as some Gram positive (*Staphylococcus*) and Gram negative (*Pseudomonas*) bacteria (Markus, 1999). As opposed to azole derivatives, PO remains fully active on resting fungal cells; its antiseptic activity proceeds from inhibition of the respiratory chain in yeast mitochondria (Bohn and Kraemer 2000). No resistance to PO has been documented to date. In addition, this antiseptic acts at low concentrations, has high affinity for keratin and is completely safe. Incorporation of PO in Allermyl® therefore aims at controlling microbial proliferation associated with allergic disease.

Specific **monosaccharides**, such as L-fucose and L-rhamnose, mimic endogenous cell-surface glycoproteins which play important biological and immunological roles in mammals. These sugars bind lectins on epidermal cell surface and have proven direct *in vitro* and *in vivo* modulatory activity on manifestations of cellular immunity (Baba et al., 1979; Stankova and Rola-Pleszczynski, 1984). Fucose and rhamnose treatment inhibits induced expression of the leucocyte adhesion molecule, ICAM-1, in activated human keratinocytes and may also be involved in the downregulation of a variety of proinflammatory cytokines, including TNF-α (Palacio et al., 1997). Overproduction of TNF-α was detected in cultures of activated canine keratinocytes (Cadiot et al., 2000) and *in vivo* in the skin of atopic dogs (Nuttal et al, 2002). Topical saccharide treatment with Allermyl® is therefore aimed at modulating the pro-inflammatory cytokine cascade and attraction of immunocompetent cells in skin. In addition, monosaccharides exhibit interesting anti-adhesive properties on the attachment of bacteria and yeasts to keratinocytes (Koch, 2000).

Vitamin E, a potent cell membrane antioxidant, was also incorported in Allermyl® to preserve long term stability of the fatty acids, and for its valuable anti-inflammatory and healing properties (Ayres, 1984).

2 Cutting-edge topical formulations

A sustained trend today is to manage canine atopic dermatitis by the use of combination therapy, including both systemic and topical medications. Most of the time topical therapy is of benefit, owing to its ability to modify events at the skin surface and bring active molecules in direct contact with the target organ, without prior systemic dilution or metabolism. Unfortunately, constraints associated with repeated use of topicals may sometimes result in failure to comply with the prescribed therapeutic protocol by dog owners.

With more than 20 years of experience in the formulation of veterinary topical products, Virbac's objective was therefore to develop anti-allergic products with immediate and deep action, long lasting effects and owner-friendly use. This commitment resulted in the development of two complementary galenic forms:

- a shampoo, for strong immediate cleansing action and removal of external pro-allergenic contributory factors from the skin surface,

- a spray lotion, leave-on formulation that increases the time of contact of active ingredients on the skin and maintains cleanliness of the treated areas .

2.1 Allermyl® shampoo: a foaming micro-emulsion

Traditional shampoos are mixtures of surfactants and water, plus hydrosoluble active molecules. They do not contain any oily phase, or very little, which impedes the introduction of liposoluble ingredients. However, Allermyl® shampoo was designed to incorporate a significant amount of hydrophobic fatty acids, as well as hydrosoluble monosaccharides and piroctone olamine. A new excipient was therefore needed to allow inclusion, in the same formula, of active ingredients with very different solubility.

An additional objective was to increase the efficiency of active ingredients by improving their distribution and diffusion in the skin.

The research conducted by Virbac resulted in a new and exclusive kind of vehicle based on micro-emulsion technology (Deroni, 1990). Traditional emulsions are complex mixtures of water, oil and tensioactives (Fig 2.). Tensioactives are molecules with both hydrophobic and hydrophilic poles. Consecutively droplets (or micellae) are formed from an oil core surrounded by a film of tensioactives. Macroscopically such emulsions present as cream, milk or lotion (depending on their viscosity), are opaque (though the droplets are quite large in size, around 1000 nm) and rather greasy. Micro-emulsions are made of much smaller droplets thanks to the addition of specific co-tensioactives with dispersing activity. A dynamic system is created where micellae are in continuous motion, resulting in more homogenous dispersion of active ingredients in the product. Presence of an oily phase allows the incorporation of fatty acids in Allermyl®, while the small size of droplets (20 nm) results in better dissolution and diffusion of active substances through the *stratum corneum*, improving their bioavailability. Micro-emulsions are transparent and non greasy. In addition, special tensioactives with high cleansing properties were selected by Virbac to obtain foaming ability.

Allermyl® shampoo was thus designed to provide rapid relief to the dog, as a consequence of the excellent distribution and penetration of its active ingredients into the skin. This provides obvious benefits in terms of efficacy and also compliance, as the time for the product to act on the animal is reduced. The shampoo demonstrates both powerful cleansing properties whilst leaving the hydrolipidic cutaneous surface film intact, thanks to its innovative galenics and oily phase. This is of paramount importance to in the removal of dirt, debris, allergens, pathogens and toxins from the skin surface, all of which are significant contributing factors to disease pathogenesis.

2.2 Allermyl® lotion: a fine fluid emulsion with non-ionic Spherulites®

Development of a complementary lotion was conducted to achieve two main objectives:
- increase therapeutic pressure, and
- facilitate performance of topical therapy by the owner.

Leave-on (“no rinse”) formulations allow active ingredients to remain longer in contact with the skin, giving a prolonged effect after application of the product. The spray form targets the lesions precisely and delivers adequate quantities of product taking into account the severity of signs on the selected area. Soaking and associated handling of the animal is avoided and facilitating application of the topical therapy. Thus the lotion can be used in the interval between two shampoos to reinforce topical treatment efficacy, or alternatively may be used to increase the

intervals between baths. For those owners that may not be able to bath the dog, the lotion also offers a practical alternative solution.

Virbac research developed a non-foaming base characterised by an oily phase finely emulsified in the aqueous phase. This emulsification was carried out with the use of well tolerated, gentle surface-active agents. The resulting non-greasy, fine, fluid emulsion allowed the incorporation of liposoluble (fatty acids) as well as hydrosoluble (monosaccharides) ingredients in the lotion. In addition it gave this new type of lotion cleansing properties, without the need for any rinsing to keep sprayed areas clean.

To increase penetration and diffusion of the monosaccharides into deeper skin structures, they were incorporated in non-ionic Spherulites®, Virbac's exclusive patented encapsulation system. Spherulites® are microvesicles made of multiple (10 to 1000) layers of surfactants that slowly break down (Fig. 3). With the breakdown of each layer, progressive release of active ingredients occurs into the skin, providing prolonged action. The surfactants used do not contain electronic charge so that the resulting microvesicules do not bind to negatively charged hair and skin (Fig. 4). Non-ionic Spherulites® therefore are able to penetrate into deep skin structures and this favours the flow of active ingredients to their site of action (Barthe et al., 1999).

In addition, persistence of active ingredients on the skin surface was enhanced in Allermyl® lotion by the use of chitosanide, a moisturizing and hygroscopic agent with adhesive and filming properties.

Deep and prolonged efficacy, as well as friendly use, are thus the hallmarks of Allermyl® lotion.

3 **Evaluation studies**

Experimental studies were conducted on this new antiallergic topical range to confirm the hypothesis underlying selection of the active ingredients and the galenic innovations.

A series of studies investigated each of the three ways by which Allermyl® was believed to provide effective action:

- Demonstration of epidermal barrier function impairment in atopic dogs and *in vivo* action of the product.
- Demonstration of the antimicrobial activity *in vivo* of piroctone olamine formulated in the shampoo.
- Demonstration of pro-inflammatory cytokine secretory competence of activated canine keratinocytes and *in vitro* inhibitory activity of monosaccharides.

In addition, evaluation of the percutaneous penetration properties of non-ionic Spherulites® in Allermyl® lotion was undertaken to substantiate galenic advantages offered by this new active ingredient vehicle.

3.1 Impact on epidermal barrier function in atopic dogs

3.1.1 Demonstration of altered epidermal barrier in atopic dogs

A pilot study was conducted by Professor Thierry Olivry of the North Carolina State University, Raleigh, USA, to determine whether dogs affected with AD exhibited altered *stratum corneum* lipids, as compared with normal dogs (Inman et al., 2001).

Punch biopsy specimens were obtained from non-lesional skin on the lateral thorax of 5 dogs diagnosed with AD and 5 clinically normal dogs. AD diagnosis was based on Willemse's criteria and exclusion of other pruritic dermatoses. Biopsy specimens were frozen and microsections were cut, mounted on slides and fixed. Post-fixation in ruthenium tetroxide was then performed to preserve and stain epidermal lipids. Thin, dehydrated sections embedded in resin were examined under the transmission electron microscope. Photomicrographs were examined in a blinded fashion by two investigators who graded the continuity and thickness of lipid depositis on a 5-point predetermined semiquantitative scale.

On photomicrographs, well-formed lipid lamellae could be identified between keratinocytes in the SC of normal dogs (photo 1). Canine SC presents a "wall-like" structure where cohesion of "bricks" of keratinocytes is provided by interspersed regular lipid deposits ("the cement"). In contrast, lipid deposition in the SC of atopic dogs was markedly heterogeneous, and many areas were even devoid of lipids. The values for both continuity and thickness of intercellular lipids were significantly lower in atopic skin, as compared to its normal counterpart.

The results of the study therefore demonstrate obvious ultrastructural defects in the SC of dogs with AD. These defects are related in particular to altered lipid lamellae deposition.

3.1.2 Correction of epidermal barrier defects with Allermyl®

A similar preliminary study was conducted by Virbac teams, using the same materials and methods as in the previous study (results not yet published). The purpose was to investigate the ability of Allermyl® topical therapy to improve SC ultrastructural defects in dogs with AD.

Non-lesional cutaneous areas were clipped on two dogs diagnosed with AD. The treatment regimen consisted of a single application of Allermyl® shampoo followed by twice daily application of the lotion for two weeks. Skin biopsies were performed before and after treatment and processed as described in the previous study.

Photomicrographic examination suggested post-treatment improvement of structural anomalies initially present in the SC of dogs with AD (photo 2). A beneficial effect of a single topical Allermyl® application could therefore be shown on the epidermal barrier function of these dogs. Additional studies are planned to further characterise the biochemical defects in SC lipids and correct them with nutraceutics and/or topical intervention.

3.2 Antimicrobial efficacy of a piroctone olamine shampoo

Topical antifungal activity of PO at in-use concentration in the shampoo was investigated on kennel dogs that were carriers of large cutaneous populations of *Malassezia pachydermatis*, at the National Veterinary School, Nantes, France (Bourdeau et al., 1999).

The beagle dogs were living under the same conditions, fed a standard diet and did not receive any other systemic or topical treatments. Twelve dogs were treated once on day 0 with the PO shampoo applied with a sponge to the ventral side of the neck. Eight additional control

dogs underwent a similar treatment procedure, but the shampoo was replaced by water. Mycological samples were obtained from relevant areas before treatment and once a day for 4 days after treatment. Samples were cultured at 32°C on modified Sabouraud's agar in Petri dishes and colony forming units (CFU) counted 7 days after incubation.

Malassezia populations were similar in both groups before treatment. A steady state in mean yeast CFU from control dogs was recorded throughout the study. In contrast, *Malassezia* populations from dogs treated with the PO shampoo decreased significantly over the study period (Fig. 5). Interestingly in the latter group, CFU decrease was not immediate but increased progressively in the days following treatment. Thus, anti-yeast activity of the product evaluated in this study was not related to a so-called "washing effect" of the shampoo but rather to a persisting antiseptic effect of PO at the skin surface.

3.3 Immunomodulating properties of monosaccharides

3.3.1 Demonstration of increased pro-inflammatory cytokine secretion by activated canine keratinocytes

Cultures of canine keratinocytes *in vitro* were developed at the Dermatology Teaching and Research Unit of the National Veterinary School of Nantes, France (Cadiot et al., 2000). The test cultures were stimulated by various concentrations of human recombinant interferon-γ (IFNγ) and lipopolysaccharide (LPS). Control cultures were preparations without keratinocytes, without stimulatory substances or Phorbol-Myristate-Acetate-stimulated keratinocytes. Supernatants in all groups were tested for TNF-α concentration after 6, 24, 48 and 72h of incubation.

IFNγ and LPS greatly increased TNF-α secretion by cultured keratinocytes, as compared to values recorded in the control groups. In this study, cellular signals such as cytokines (IFNγ) or bacterial surface antigens (LPS) produced an activation of canine keratinocytes resulting in marked over-secretion of the pro-inflammatory cytokine, TNF-α.

3.3.2 Inhibition of cytokine secretion by monosaccharides

Using the same *in vitro* keratinocyte activation model as described above, a subsequent study was designed to evaluate the inhibitory activity of monosaccharides on keratinocyte TNF-α release (Ibisch et al., 2001).

Activated keratinocytes in culture were treated with fucose, rhamnose and a combination of both. Monosaccharide activity was evaluated at concentrations corresponding to those used in Allermyl® products. Other control activated keratinocyte cultures were exposed to a potent anti-inflammatory agent, dexamethasone, at a concentration proven to have inhibitory activity on cytokine-release by macrophages.

Comparable relative reduction of TNF-α release by keratinocytes was recorded in all groups, ranging from 56% in the cultures exposed to dexamethasone to 76% in the cultures exposed to the fucose - rhamnose combination (Fig. 6). No reduction of the pro-inflammatory cytokine secretion was recorded in cultures left unexposed to the anti-inflammatory agents.

Monosaccharides therefore demonstrate immunomodulating activity on signals that mediate cell immunity and which may play a significant role in the initiation and maintenance of the inflammatory state in the skin (refer also to Jacqueline Viac's presentation in this issue: "The Activated Keratinocyte").

3.4 Bio-distribution of non-ionic Spherulites® in dog skin

Radioimaging techniques were used to investigate the percutaneous diffusion of the encapsulating system used in the lotion (Barthe et al., 1999).

Eight epidermal biopsy specimens were obtained from healthy dog skin. A suspension of radioactive probe (14C-Palmitic acid) encapsulated in non-ionic Spherulites® was dropped onto 5 test specimens. Three control biopsy specimens were exposed to the non-encapsulated radioactive probe, to non-labelled Spherulites® or remained untreated, respectively. All specimens were rinsed then air-dried. Biopsies were frozen, sections cut and placed on glass slides for histoautoradiographic examination. Other sections were submitted for direct radioactive quantification using a high resolution radioimaging device (micro-imager, Biospace-Mesures, Paris).

In sections from biopsies treated with the non-encapsulated probe, the silver grains were distributed mainly in the superficial cellular layers of the epidermis. Very little radioactivity was detected in hair follicles. By contrast, in sections from biopsies treated with the Spherulite®-encapsulated probe, silver grains were abundant in all epidermal cell layers, within hair follicles and follicular infundibula, and in the dermis (photo 3).

Direct quantification of radioactivity by micro-imaging confirmed that the signal with the non-encapsulated probe was distributed at the skin surface, while the radioactivity signal with the encapsulated probe was increased in the deeper parts of the biopsy section. No radioactivity was detected in the frozen biopsy treated with unlabelled Spherulites® and in the untreated frozen biopsy.

These results indicate that non-ionic Spherulites® promote the diffusion of the radioactive probe into the skin and thus confirm the usefulness of this microvesicle vehicle technology to enhance the delivery of encapsulated active substances through epidermal cell layers, into cutaneous appendages (hair follicles) and into the dermis.

3.5 An ongoing evaluation process

Evaluation of feedback from users and confirmation of product tolerance under field conditions were then conducted with both Allermyl® shampoo and lotion. The products were tested in veterinary clinics on allergic dogs, with no concomitant parasitic infestation or cutaneous infection.

The treatment regimen in a the first series of studies combined a single bath with Allermyl® shampoo, followed by twice-daily application of Allermyl® lotion for 2 weeks. The 14 dogs did not receive any other treatment. Pruritus and erythema were reduced over the study period in 86% and 79% of the dogs, respectively, according to veterinarians. Owners' perception of efficacy was similar, with 86% of owners satisfied at the end of the treatment period.

A second series of studies was conducted to evaluate the short-term efficacy of Allermyl® lotion on 68 pruritic dogs, diagnosed with allergy. Ten minutes after application of the lotion, veterinarians reported a perceptible reduction in erythema intensity for 56% of the cases. Within three days of twice-daily lotion application at home, owners detected a decrease in the frequency and intensity of scratching/licking for 88% of the dogs, and a reduction in skin redness for 82% of the cases.

In both of these preliminary field studies tolerance of the tested products proved excellent, under real conditions of use.

Additional evaluation of Allermyl® range efficacy is presently underway. Controlled clinical field trials on dogs diagnosed with confirmed atopic dermatitis are being conducted on a broad international scale.

Development of new concepts (new ingredients and galenics, muti-targeting action) based on updated knowledge on disease pathogenesis, testing using modern investigation techniques (electron microscopic observation of canine *stratum corneum*, study of canine keratinocyte cytokine secretion), collaboration with world specialists in universities and public research agencies to bring en ever continuing flow of scientific evidence to prescribing practitioners: this was the story of Allermyl® development. A never-ending story indeed.

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