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## Temporal changes in the granulocytic responses to experimental infection of the skin of mice and sheep with *Dermatophilus congolensis*

LLOYD (D.H.), SASIAK (A.B.), KITSON (S.), McEWAN JENKINSON (D.), ELDER (H.Y.). Changements temporels des réponses granulocytaires à l'infection expérimentale de la peau de souris et de mouton avec *Dermatophilus congolensis*. *Revue Élev. Méd. vét. Pays trop.*, 1993, 46 (1-2) : 271-276

Les types de réponses cellulaires inflammatoires de la peau à l'infection avec *Dermatophilus congolensis* ont été déterminés chez des souris et des moutons à partir de prélèvements histologiques pris avant et à intervalles, après l'application topique de zoospores infectieuses sur la peau frottée à l'éther. Des cellules neutrophiles, éosinophiles, basophiles et des mastocytes ont été identifiées par coloration histo-chimique. Les changements temporels des cellules B, T et les populations de cellules MHC classe II<sup>+</sup> dendritiques font l'objet d'un rapport distinct. Les stades filamenteux de la bactérie ont été observés dans le stratum corneum des deux espèces ; chez le mouton, ils se trouvaient également dans les couches extérieures de l'épiderme vivant. Chez les deux espèces, de grands nombres de neutrophiles et quelques lymphocytes pénétraient l'épiderme et entraient dans la zone de surface infectée. Dans le derme sous-adjacent, il y avait une accumulation de cellules dendritiques immédiatement sous l'épiderme infecté et l'on pouvait constater une dégranulation des mastocytes ; les basophiles et les éosinophiles ne semblaient pas activement impliqués. La différence frappante entre les deux espèces était la durée de l'infection et la réponse associée, qui avait une durée de 5 jours environ chez la souris contre plus de 21 jours chez le mouton. Le nombre de neutrophiles chez la souris était par exemple élevé après 12 h et avait atteint son maximum 60 h après l'infection, tandis que le maximum chez le mouton n'était atteint qu'après 120 h.

*Mots clés* : Ovin - Souris - *Dermatophilus congolensis* - Infection expérimentale - Histopathologie - Peau - Granulocyte - Mastocyte.

### INTRODUCTION

*Dermatophilus congolensis* normally invades only the epidermis and provokes a predominantly neutrophilic response with epidermal hyperplasia. Recruitment of mononuclear cells occurs much later and is much less marked (8, 10). Susceptibility to infection varies substantially between species. Mice are particularly resistant, the neutrophilic response peaks early and lesions resolve in about 7 days (6, 10) whilst sheep are highly susceptible ; neutrophil responses peak at 10-12 days and healing may take as long as 38 days (1).

The reasons for the differences in resistance and the duration of lesions between species remain largely unknown. Comparative studies have shown that rabbit neutrophils are more efficient at killing *Dermatophilus* zoospores than those of sheep and guinea-pigs (10). However, no direct comparison has been made between sheep and mice to determine the reasons for the differences in their responses to infection. This study was designed to investigate and compare the cellular responses to infection in murine and ovine skin on a quantitative basis to assist in evaluation of the role of specific and non-specific immune mechanisms in protection against and recovery from dermatophilosis.

### MATERIALS AND METHODS

#### Inoculation and sampling

##### Inoculum

Motile zoospores were harvested from 48 h aerobic blood agar cultures of *Dermatophilus congolensis* strain SS18C and suspended in sterile peptone water to give a concentration of 10<sup>8</sup> to 10<sup>9</sup> per ml, as described by HOW and LLOYD (3). Sterile peptone water was used as the control medium.

##### Mice

Groups of 4 randomly-selected, male and female BALB/c mice, aged 9-10 months, were housed separately with free access to water and a commercial rodent diet. A site (16 cm<sup>2</sup>) on the dorsal skin of the trunk of each mouse was clipped (Oster clippers, 40 gauge blades) and swabbed with ether-soaked cotton-wool. Zoospores of *D. congolensis* were applied to each site by dipping a sterile cotton-wool swab into the inoculum and rolling it over the inoculation site for one min. Half-an-hour after inoculation all members of one group were killed by ether inhalation and a skin sample was taken from the treated skin of each of them. Skin samples were obtained in the same way from the inoculated skin of other groups of four mice at 12 h intervals thereafter, up to 120 h after inoculation. Skin samples were also obtained, after 1/2, 24, 48 and 72 h, from groups of four mice which had been clipped, ether swabbed and inoculated with sterile peptone water.

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## Sheep

Ten 4-month-old Suffolk lambs were housed on straw, in two groups of five, with access *ad libitum* to water, hay and concentrates. On one group, ten sites, each 16 cm<sup>2</sup>, were selected at random on the dorsal skin of the trunk of each animal. The sites were clipped, ether swabbed and inoculated with zoospores as described above. Skin samples were taken under local anaesthesia using a 6 mm biopsy from one site on each animal before (0h) and at 6, 12, 24, 48, 72, 120 h and at 9, 15 and 21 days after infection.

On the other group, three sites were selected and treated in the same way except that sterile peptone water was applied instead of the inoculum. Skin samples were obtained as described from each animal before and at 1/2 and 24 h, and at 9 days after treatment.

## Histology

Skin samples were fixed in modified Bouin's fixative at room temperature and processed to paraffin wax using the St. Marie technique (9). Serial sections were cut at 7 µm from each block. Groups of five sections, taken at intervals of at least 50 µm, were mounted on slides pre-treated with poly-1-lysine. This enabled visualisation and enumeration of each cell type under consideration, on five sections at every time interval. Neutrophils, basophils and mast cells were studied following staining with Giemsa and eosinophils after staining with carbol-chromotrope (5). Cells of dendritic morphology were counted on haematoxylin and eosin and Giemsa stained sections.

## Quantification and analysis

Cells were counted within an area of 0.021 mm<sup>2</sup> of dermis in all five sections from each block, a) under and b) adjacent to the lesion and the data were compared by analyses of variance.

# RESULTS

## Clinical observations

### Mice

A faint erythematous reaction was visible on infected skin after 24 h. At 48 h raised, focal or confluent areas of scab formation, accompanied by erythema and slight swelling, were present. These scabs persisted until 84 h but were then progressively shed and at 96 h only a few foci of thin scabs could be found. At 120 h, no lesions were visible

but the skin was alopecic in previously affected areas. No lesions developed at the control sites of mice treated with peptone water.

### Sheep

Signs of oedema were present at inoculated sites 24 h after infection and, in some instances, a glistening exudate could be seen. Crusting and scab formation was evident at all sites by 4 days and peaked at 15 days. Scabs were firmly attached to the skin surface at that time but subsequently became progressively detached. A few detached scabs still remained attached to the growing fleece at some sites at 21 days.

## Histopathology

### Mice

In unstimulated skin, mast cells were prominent in the vicinity of blood vessels, particularly around hair follicles, and polymorphonuclear cells and lymphocytes were occasionally located within blood vessels. At 12 h the only observable change was evidence of mast cell degranulation in the zone between the sebaceous gland and the hair bulb, but mainly beneath the muscle layer (photo 1). By 24 h, scabs containing filaments of the bacterium were present at the surface. The epidermis at these locations was hypertrophied but intact, although foci of degeneration in the outer living layers were observed in places, cells of dendritic morphology were observed accumulating within the dermis immediately under the infected epidermis. There was also evidence of inflammatory cell infiltration of the dermis below the dendritic cell foci, with a predominance of neutrophils. By 36 h the sub-epidermal aggregation of dendritic cells was more intense and the dermal infiltration of neutrophils has increased. However, few were located within the now markedly hypertrophied epidermis, although neutrophils were present in the scabs. Mast cell degranulation was now prominent, particularly underlying the subdermal muscle. At 48 h there was marked dermal infiltration by neutrophils and some lymphocytes which accumulated under the epidermis within the zone of dendritic cells and were present in the hypertrophied epidermis, especially within vesicles which had formed in the outer stratum spinosum. The larger of these vesicles, which were packed with neutrophils and contained lymphocytes, lay under extensive surface scabs within which filaments of the bacterium were prominent above a layer of neutrophils. The lower layers of the epidermis remained intact. There was still evidence of mast cell degranulation at this stage. The picture at 60 h was similar to that at 48 h although the mast cell degranulation was less evident. By 72 h, dendritic cells were still prominent under the epidermis which was still slightly hypertrophied but no longer exhibited marked vesicula-



Photo 1 : Degranulating mast cells beneath the subcutaneous muscle layer of the mouse at 48 h.

tion. Scabs, each containing a zone of bacterial filaments above a layer of neutrophils, were located at the surface overlying two to three keratin layers ; in places, two layers of filaments interspersed with neutrophils were seen. Filaments were still traced within the outer layer of the stratum corneum at some locations. The dermal infiltrate was still predominantly of neutrophils, but none was traced within the epidermis. Mast cell degranulation was not observed. This situation persisted until 84 h but by 96 h, most of the skin resembled the uninfected controls, although a few foci of thicker epidermis with underlying dendritic cells and some neutrophils were still present. By 108 h only two small foci, where underlying dendritic cells and neutrophils were present, were located and by 120 h the skin was indistinguishable from uninfected controls.

### Sheep

In untreated sheep skin, mast cells and dendritic cells were prominent around blood vessels and were particularly noticeable in the neighbourhood of the hair follicle units. Other cell types were only occasionally located

within blood vessels. At 6 h the *bacterium* was not detected but there were signs of a host response to the challenge. There was evidence of foci of dendritic cell accumulation just under the epidermis and of mast cell degranulation. Lymphocytes had infiltrated into the dermis but few neutrophils were found outside the blood vessels. The epidermis exhibited no signs of abnormality. Between 12 and 120 h the dendritic cell accumulation at specific foci increased and neutrophil numbers within the dermis generally, but particularly at these foci, gradually rose. Lymphocytes were also present at these foci but there was no evidence of penetration of the epidermis by any of the invading cell types nor signs of the organism at the surface. Degranulating mast cells were regularly found within the dermis. At 9 days the epidermis was considerably hypertrophied and thick laminated scabs containing numerous alternating layers of bacterial filaments and densely packed neutrophils were present at the surface. In places, much of the stratum spinosum was vesiculated to the level of 1 to 2 cells above the basal lamina ; the outer vesicles were generally large and contained neutrophils and lymphocytes (photo 2) whereas those deeper within the epidermis were smaller although also full of the

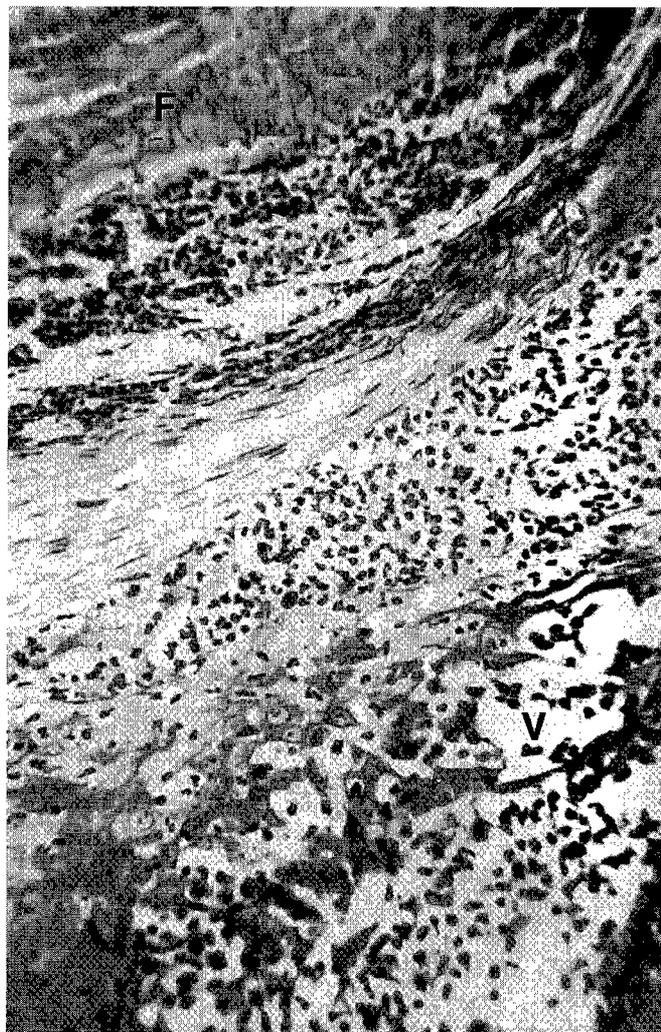


Photo 2 : Invasion of the degenerative ovine epidermis after 9 days by neutrophils in response to invasion by *D. congolensis*. Filaments (F) of *D. congolensis* are present in the scab and the formation of vesicles (V) containing neutrophils and lymphocytes can be seen.

same cell types which had generally infiltrated the epidermis. A dense dendritic cell aggregation was present immediately under the epidermis and lymphocytes and neutrophils were prominent in this region. The mast cells which were detected within the dermal infiltrate were markedly degranulated although those located between foci of infection appeared to be normal. The picture at 15 days was similar to that seen at 9 days although in places the vesicles within the epidermis extended to the basal lamina; there the dendritic cell aggregation was particularly dense. The surface scabs were often 2 to 3 times thicker than the hypertrophied epidermis. By 21 days, some laminated scabs were still detected at the skin surface, but the thickness of the epidermis had returned to control levels with the exception of a few places where slight hyperplasia was still present. The dermal blood vessels

appeared to be dilated and contained neutrophils and lymphocytes; these cell types were, however, also observed outside the vessels throughout the dermis. The distribution of dendritic cells resembled that of untreated skin and the mast cells had a normal appearance.

## Patterns of cellular response

### Neutrophils

In the mouse, dermal neutrophil numbers under the lesion had risen significantly by 24 h ( $p < 0.01$ ) (fig. 1) and continued to rise until 60 h ( $p < 0.01$ ) when it gradually fell until 96 h. There was a second rise between 96 and 108 h ( $p < 0.001$ ) compared with pre-inoculation levels. Adjacent to the lesion there was a significant rise in dermal neutrophil count between 24 and 48 h ( $p < 0.001$ ).

In the sheep, the number of neutrophils in the dermis also rose significantly within the first 30 min ( $p < 0.001$ ) and progressed to a plateau level by 72 h ( $p < 0.001$ ) except for a slight, but significant, fall between 6 and 12 h ( $p < 0.01$ ) (fig. 1). This level was maintained until 15 days but the number had begun to fall by 21 days ( $p < 0.01$ ). During this latter period, the neutrophils were most often present in or around blood vessels. The pattern of neutrophil influx adjacent to the lesion was similar although the total numbers were only about 10 per cent of those found under the lesion.

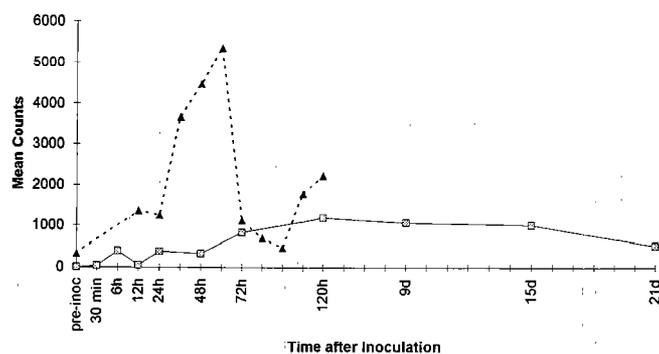


Figure 1 : Temporal changes in the number of neutrophils beneath the lesion in mice ( $\Delta$ ) and sheep ( $\square$ ). Mean cell counts are given along the y axis and the time in hours (h) and days (d) after inoculation, along the x axis.

### Eosinophils and basophils

In both species, eosinophils were only found within blood vessels and this cell type did not vary significantly in number as a result of the infection. Eosinophil number/mm<sup>2</sup> ranged from 4.90 - 44.12 in the mouse and from 1.96 - 43.14 in the sheep. Basophils were not detected in any of

the samples of mouse skin and in the sheep those present (range 1.96-21.55/mm<sup>2</sup>) were also found only within blood vessels; ovine basophil number did not change significantly during infection. The observed variations in eosinophil and basophil number thus mainly reflected the numbers of blood vessels present in the randomly selected fields of study.

### Mast cells

Mast cell number in the mouse, which ranged from 44.12-174.02/mm<sup>2</sup>, rose significantly under the lesion between 24 and 48 h ( $p < 0.001$ ) and remained elevated until 96 h. The number had fallen to prestimulation levels by 108 h. Between 12 and 60 h many of the cells counted exhibited signs of degranulation. Adjacent to the lesion there was no significant change in cell appearance and number. Mast cell number in the sheep ranged from 100.00-178.43/mm<sup>2</sup> and did not vary significantly with infection. However, mast cell degranulation was evident in the skin between 6 h and 15 days.

## DISCUSSION

This work has confirmed the oft-repeated observation that neutrophils are the most abundant cells at the site of *Dermatophilus* infection (1, 10). It has also shown differences and similarities between 2 mammalian species, one resistant and one susceptible, in the amount and duration of the neutrophil influx, following similar experimental infections. The level and duration of granulocytic responses to the organism reflect the differences in lesion duration seen in the mice and sheep.

These detailed temporal studies have demonstrated for the first time the links between neutrophil recruitment to the site of infection and mast cell degranulation, which precede the appearance of clinical signs of infection, in both of the species studied.

The first phase of neutrophil recruitment to the site of infection occurred in the absence of any clinical or histological signs of infection, such as erythema or the presence of *D. congolensis* in histological specimens. This initial peak of neutrophil influx coincided with the first observations of mast cell degranulation in both species. It is not possible to state with any certainty whether the initial recruitment of neutrophils occurred because of mast cell degranulation around the blood vessels in the dermis, or whether it had already started and the involvement of mast cells led to the boost in neutrophil numbers seen later. There are inflammatory and immunologically mediated pathways which can lead to neutrophil adhesion to, and migration across, the endothelium which can be initiated by keratinocytes. Thus it is possible to speculate that damage to epidermal cells by *Dermatophilus* could cause them to release IL-1 and TNF $\alpha$ , which could then

act directly upon endothelial cells of local blood vessels to upregulate adhesion molecule ligands such as ICAM-1, IL-8 and E-selectin leading to the trapping and recruitment of passing neutrophils. These neutrophils would then be attracted to the site of infection along a chemotactic gradient featuring IL-8 produced by the keratinocytes and by dermal fibroblasts, following IL-1 and TNF $\alpha$  stimulation. The degranulating mast cells around the blood vessels in the deep dermis would further contribute to neutrophil recruitment by histamine release leading to expression of other adhesion molecule ligands, such as platelet activating factor (PAF) and P-selectin on the endothelial cells (11). The mechanism which initiated the degranulation of mast cells remains unknown but may have been the generation of complement components C3a and C5a by the action of bacterial enzymes or surface components on the alternative complement pathway (7).

Such a dual-phase mechanism may help to explain the next temporal event which was a quiescent period when neutrophil numbers plateaued or fell. The end of this lag period coincided, in both species, with the clinical and histological appearance of lesions caused by *Dermatophilus*. After this, the numbers of neutrophils under the lesions rose to peaks which coincided with the peak of lesion activity in both species. As the lesions then healed, mast cell degranulation ceased to be observed and neutrophil numbers fell. The localisation under the lesion of the mast cell degranulation seen in the histological sections suggests that the phenomenon is mediated by factors which operate in tightly defined areas.

The main difference between the species was in the duration of the experimental infection and of the immune response, which was far longer in the sheep than in the mice. However, five times as many neutrophils were present under the infected sites of the mice when compared with those of the sheep and this may have been a factor in the shorter duration of lesions in the mice. The reason for the more effective recruitment of neutrophils in the mouse remains unknown.

In this study, eosinophils and basophils did not appear to have any role in the response to *D. congolensis*, this is in contrast to the situation seen in other infections involving the epidermis. Increased numbers of basophils accumulate at the site of experimental orf virus infection during the late phase of the response (4) and the role of eosinophils in the cutaneous reaction to epidermal and dermal damage from arthropod bites is well documented (2).

The most important finding from this study was the link between mast cell degranulation and neutrophil recruitment to the site of infection before any signs of clinically observable infection, either at the skin surface or in histological sections. Clearly, something is happening at a cellular level before *D. congolensis* becomes manifest in the epidermis and studies of the reactions to *Dermatophilus* which take place before 6 hours may be indicated.

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The regulation of the influx of neutrophils to the site of experimental *D. congolensis* infection is only part of the story. Detailed temporal studies of the mononuclear and dendritic cell reaction to *D. congolensis* infection in sheep and mice are in progress and will be reported elsewhere.

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The patterns of dermal inflammatory cell response to infection with *Dermatophilosis congolensis* were determined in mice and sheep from histological samples taken before and at intervals after topical application of infective zoospores to ether-swabbed skin. Neutrophils, eosinophils, basophils and mast cells were identified by histochemical staining. Temporal changes in the B cell, T cell, and MHC Class II<sup>+</sup> dendritic cell populations form part of a separate report. The filamentous stages of the bacterium were observed in the stratum corneum of both species ; in the sheep they were also found in the outer layers of the living epidermis. In both species, large numbers of neutrophils and some lymphocytes penetrated the epidermis and entered the infected surface region. Within the underlying dermis there was an accumulation of dendritic cells immediately below the infected epidermis and evidence of mast cell degranulation ; the basophils and eosinophils did not appear to be actively involved. The striking difference between the two species was the duration of the infection and the associated response which, in the mouse, lasted about five days in comparison with over 21 days in the sheep. Neutrophil numbers in the mouse for example were elevated by 12 h and had peaked at 60h after infection, while in the sheep they did not peak until about 120 h.

**Key words :** Sheep - Mice - *Dermatophilus congolensis* - Experimental infection - Histopathology - Skin - Granulocyte - Mast cell.

Se determinaron los patrones de la respuesta celular inflamatoria de la piel a *Dermatophilus congolensis* en ratones y ovejas. Se utilizaron muestras histológicas obtenidas antes y después de una aplicación tópica de zoosporas infectivas, sobre una piel previamente tratada con éter. Mediante tinciones histoquímicas se identificaron neutrófilos, eosinófilos, basófilos y mastocitos. Los cambios temporales sufridos por las células B, las células T y las poblaciones de dendritas MHC clase II<sup>+</sup>, se describen en un artículo aparte. Los estadios filamentosos de la bacteria se observaron en el estrato córneo de ambas especies, observándose también en las capas superficiales de la epidermis de ovejas. En ambas especies los neutrófilos y algunos linfocitos penetraron la epidermis hasta la zona infectada. Dentro de la dermis profunda, se produjo una acumulación de células dendríticas, inmediatamente bajo la epidermis infectada, con evidencia de desgranulación de los mastocitos. Los basófilos y los eosinófilos no aparecieron activamente involucrados en este fenómeno. La gran diferencia entre las dos especies fue la duración de la infección y la respuesta asociada. En ratones tuvo una duración aproximada de cinco días, contra 21 días en la oveja. La cantidad de neutrófilos en el ratón se mantuvo elevada durante 12 horas, con un pico 60 horas post-infección, mientras que en la oveja el pico se dió a las 120 horas.

**Palabras claves :** Ovino - Ratón - *Dermatophilus congolensis* - Infección experimental - Histopatología - Piel - Granulocito - Célula fibrosa.