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Experimental dermatophilosis in murine models of immunodeficiency

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Des souris gnotobiotiques ayant des déficiences immunitaires congénitales ont été infectées avec *Dermatophilus congolensis*, agent pathogène cutané. Des souris sans thymus (nues), avec une déficience en cellules T, se sont montrées moins sensibles que des souris nues qui portaient également la mutation beige (bg/nu) ayant des défauts de cellules tueuses et de granulocytes, l'équivalent murin du syndrome de Chediak-Higashi. La présence additionnelle chez d'autres souris avec la mutation beige, du gène d'immunodéficience lié au chromosome X, qui cause une réduction de la réponse des cellules B, n'a pas augmenté la sensibilité. Des souris BALB/c possédant la mutation nue et montrant une déficience de macrophages, avaient un niveau modéré de sensibilité, plus élevé que celui de souris nues non consanguines mais moins que celui des souris beiges-nues. Les lésions sur les souris à poils avaient un aspect différent de celles sur les souris nues (nu et bg/nu). Sur les souris à poils, des croûtes minces se développaient et guérissaient rapidement, tandis que les lésions sur les souris nues commençaient comme des nodules et se changeaient ensuite en croûtes. Les souris nues BALB/c développaient des lésions atypiques, ressemblant à des ulcères. Des souris axéniques nues et beiges-nues ont montré les mêmes types et mêmes durées d'infection que les animaux gnotobiotiques, ce qui suggère que l'intervention par des bactéries, d'une flore cutanée limitée, ne jouait pas de rôle majeur dans la défense contre *D. congolensis*. Néanmoins, une analyse bactériologique a montré que *D. congolensis* pouvait survivre dans l'intestin de souris axéniques. Ce travail accentue l'importance de mécanismes immunitaires non-spécifiques dans la résistance à *D. congolensis*, tels que l'hyperprolifération épidermique et le neutrophile.

Mots clés : Souris - Dermatophilose - *Dermatophilus congolensis* - Infection expérimentale - Immunodéficience - Lésion - Maladie de la peau - Gène - Résistance aux maladies.

INTRODUCTION

Successful infection by the epidermal pathogen *Dermatophilus congolensis* is a complex process dependent on various factors including the host immune response and the presence of an inhibitory bacterial skin flora (10). The immune response in dermatophilosis is mediated mainly by neutrophils (14, SASIAK and JENKINSON, in preparation). Specific immune responses have also been shown to play a part and T lymphocytes are known to accumulate under the site of infection (1, 3) but their role in the healing process is not known. In the first experiment described in this study, the relative importance of T cells in resistance to experimental *D. congolensis* infection, *in vivo,* was studied by the use of congenitally athymic mice.

Environmental competition between commensal and pathogenic bacteria is one of the factors which can affect disease severity. In the natural state, infection with *D. congolensis* does not occur in isolation and, in trying to infect, the organism has to compete with the other bacteria and yeasts which have already colonized the skin. Germ-free mice provide a means of examining whether the presence of other organisms can affect the infectivity of *D. congolensis*. In the second experiment, germ-free, immunodeficient mice were infected with *D. congolensis* and the course of the resulting lesions studied.

MATERIALS AND METHODS

Animals

Gnotobiotic (known, limited bacterial flora) and germ-free mice with quantitative or functional deficiencies of various populations of immune cells were used in both experiments (table I). All mice, except for the inbred BALB/c nude group, were bred from a random genetic background. During each experiment, all the animals were housed in a single isolator.

Bacteriology

The presence of existing skin, faecal and environmental organisms was monitored using standard bacteriological techniques. Swab samples were taken from the floor of the isolator and from the skin of several mice ; faecal samples were also examined at the start and finish of each experiment. The germ-free faecal samples were incubated in nutrient broth and in cooked meat medium. Positive samples were cultured on blood agar. Swabs from the gnotobiotic isolator were either processed immediately or left overnight at room temperature in transport medium before being cultured on aerobically and anaerobically on blood agar plates, at 37 °C. Identification of any positive samples was carried out to species level, where possible, using conventional techniques supplemented by the API system (Bio-Mérieux).

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TABLE I Types of mice used.

Experiment 1 : Gnotobiotic mice		
Mice	n	Immune deficiency
nu+	5	Normal control, heterozygote with nude (nu) gene.
nu/nu	5	Homozygote for nude gene ; athymic.
nu/nu-bg/bg	5	Athymic with additional functional neutrophil and NK cell deficiency, and Chediak-Higashi-like syndrome given by beige (bg) gene.
nu/nu-bg/bg-xid	5	As above, with T-independent B cell functional deficiency from x-linked immunodeficiency (xid) gene.
BALB/c-nu/nu	5	Athymic with functional macrophage deficiency.
Experiment 2 : Germ-free mice		
Mice	n	Immune deficiency
nu+	4	Normal control, heterozygote with nude (nu) gene.
nu/nu	4	Homozygote for nude gene ; athymic.
nu/nu-bg/bg	3	Athymic with additional functional neutrophil and NK cell deficiency,and Chediak-Higashi-like syndrome given by beige (bg) gene.

Inoculation procedure

The mice were inoculated with motile zoospores of a Scottish ovine isolate (SS18C) of *D. congolensis*. The zoospore suspensions were prepared as described by HOW and LLOYD (7) and were checked for motility by microscopic examination. The concentrations and purity of the inocula were monitored by a spread plate method. After use in the isolator, the residual inocula were again checked for viability by culture on blood agar.

The hair was clipped only from the backs of the haired mice in experiment 1. The clipping of the mice required several passes of the clipper blades. This did not cause any visible trauma but may have altered the skin surface equilibrium by plucking the hair rather than by cutting it. The skin of the inoculated sites, on all the mice, was swabbed with ether before inoculation. *Dermatophilus* was applied to the skin by dipping a sterile cotton-wool swab into the zoospore suspension and rolling it over the skin for one minute.

Observation and scoring of lesions

The appearance of the skin and of any lesions present was recorded for up to 10 days after inoculation. Lesions that appeared at the inoculated sites were scored for severity on a scale of one to four (0, normal skin; 1, small focal lesions, slight inflammation ; 2, focal lesions affecting less than 50 % of the inoculated area, moderate inflammation ; 3, semi-confluent areas of infection [involving more than 50 % of inoculated area], severe inflammation ; 4, confluent infection, any spread of infection outside the infected area, severe inflammation).

Histopathology

Skin samples were taken aseptically, from euthanased, infected mice, at the end of the observation period. A further set of samples was taken from uninfected mice, both gnotobiotic and germ-free, as controls.

The skin on the back was excised, mounted on card, fixed in modified Bouin's fixative and processed for paraffin wax embedding. Sections were cut and stained with haematoxylin and eosin (H & E), Giemsa, Methyl-green-Pyronine (for plasma cells) and Gram's method.

Statistics

The differences between the mean lesion scores from the different groups, in both experiments, were examined by means of Student's t test.

RESULTS

Bacteriology

Experiment 1 : Gnotobiotic mice

The bacterial flora on the skins of the gnotobiotic mice consisted mainly of *Streptococcus* sp. *(Streptococcus faecalis* and another unidentified *Streptococcus)* together with *Lactobacillus plantarum, Bacillus macerans* and a catalase positive, DNAase and coagulase negative, *Staphylococcus* sp. Isolator surface sampling showed only colonies of a *Streptococcus* sp. No organisms were isolated from skin swab samples from the haired mice either before or after inoculation. At the end of the gnotobiotic experiment, swab samples taken from the lesion sites on nu/nu-bg/bg and nu/nu-bg/bg-xid mice revealed the presence of *D. congolensis* and a *Streptococcus* sp.

Experiment 2 : Germ-free mice

No organisms were present in faecal samples from the germ-free mice at the beginning of the experiment but *D. congolensis* was isolated from faecal samples at the end. No organisms were isolated from skin swab samples at the end of the germ-free experiment.

Monitoring of the inocula after removal from the isolators showed that viability had been maintained. Semi-quantita-

tive assessment of inoculated blood plates and colony counting of the inocula in the 2nd experiment indicated that no significant loss of viability had occurred. In the first experiment, one of the aliquots of inocula became contaminated with *Streptococcus* sp. from the mouse skin during the inoculation procedure.

The concentration of zoospores in the inoculum in the first experiment was 7 x 10^{12} cpu/ml and in the second, 4 x 10^{4} cpu/ml.

Clinical observations and lesion scores

Experiment 1 : Gnotobiotic mice

The time course and appearance of the lesions on the skin of the haired and hairless mice were different (table II). The haired mice developed thin crusts, with no visible inflammation, which detached from the skin by 4 days after inoculation. Hair then began to slowly regenerate in the alopecic areas after the scabs had fallen.

TABLE II Patterns of lesion development on mice in Experiment 1.

Day	Nos. Affected	Clinical observations
Day 1		
nu+	0/5	Normal skin
nu/nu	1/5	Slight nodules on one animal only.
nu/nu-bg/bg	1/5	Slight nodules on one animal only.
nu/nu-bg/bg-xid	1/5	Slight nodules on one animal only.
BALB/c-nu/nu	0/5	Normal skin
Day 3		
nu+	5/5	Extensive, thin crusts on all mice.
nu/nu	3/5	Small nodules visible.
nu/nu-bg/bg	5/5	Larger areas of nodules and skin thickening.
nu/nu-bg/bg-xid	5/5	Large areas of nodules turning into crusts.
BALB/c-nu/nu	5/5	Small nodules present.
Day 7		
nu+	2/5	Lesion healing, scabs falling or reduced.
nu/nu	2/5	Almost negative, 1 mouse has hairless patches.
nu/nu-bg/bg	5/5	Crusted lesions beginning to heal.
nu/nu-bg/bg-xid	4/5	Crusted lesions beginning to heal.
BALB/c-nu/nu	3/5	Ulcer-like lesions appear.
Day 10		
nu+	0/4	No. scabs remaining, hair regrowth started.
nu/nu	1/4	Small nodules on one mouse only.
nu/nu-bg/bg	4/4	Healing scabs.
nu/nu-bg/bg-xid	3/4	Healing scabs.
BALB/c-nu/nu	3/4	Healing lesions.

The hairless mice developed nodules at the site of inoculation which were surrounded by areas of inflammation and skin thickening. When the nodules developed into crusts these tended to have raised margins. The above stages in the progression of a severe lesion in one of the mice in the nu/nu-bg/bg-xid group can be seen in photos 1 to 3. At 3 days after inoculation the skin on the back

Photo 1 : Development of a lesion on a mouse of the nu/nu-bg/bg-xid group. At 3 days after inoculation the skin is thickened and erythematous. Nodules have appeared.

Photo 2 : The same lesion at 4 days after inoculation, a thick scab has formed.

Photo 3 : By 7 days after inoculation, the lesion is healing and the scab is getting smaller.

Photo 4 : Unusual ulcer-like lesions seen on one of the BALB/c nude mice 7 days after inoculation.

was thickened and reddened (photo 1) and by 4 days had begun to show crusting (photo 2). After 7 days the lesion was healing and the scab was smaller, surrounded by a raised margin (photo 3).

The BALB/c-nu/nu mice developed atypical lesions which began as barely visible nodules which then went on to become open ulcers with raised margins, by 7 days after infection (photo 4).

There were significant differences in the severity of lesion scores between the groups of mice. By 3 days after infection the group mean score of the haired (nu+) mice was significantly higher than that of those in all the other groups ($p < 0.001$). At this time the nu/nu mice had the lowest lesion scores of all the groups ($p < 0.01$). By 7 days afler infection the nu/nu-bg/bg mice had the highest lesion scores of all the groups ($p < 0.01$) and the nu/nu group mice had the lowest ($p < 0.01$). There was no difference between the scores from the nu/nu and nu+ groups at 7 days after infection (fig. 1).

Towards the end of the experiment, one of the nu/nu group developed small patches at the inoculated site where it appeared that the residual hair had fallen out. These patches went on to become small crusts and may have represented a sub-clinical or delayed infection.

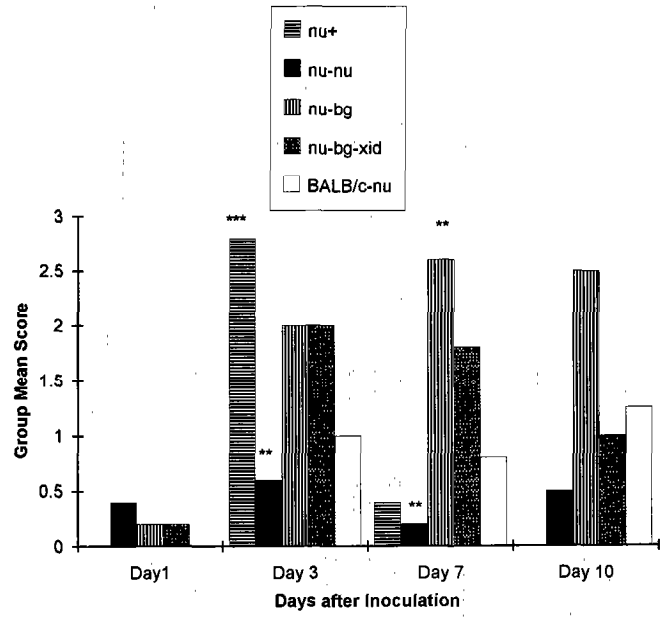

*Figure 1 : The patterns of lesion development and persistence in the groups of normal and immunodeficient gnotobiotic mice. The groups of haired mice (nu+), nude mice (nu/nu), nude-beige mice (nu/nu-bg/bg), nude-beige-B-cell deficient mice (nu/nu-bg/bg-xid) and BALB/c- nude mice (BALB/c-nu/nu) are shown along the x axis. Each group was observed for 10 days after infection and the columns represent the various days post-infection. Blank spaces indicate a zero score on a particular day (e.g. for the nu+ group on days 1 and 10). (***= $p < 0.001$, **= $p < 0.01$.)*

Experiment 2 : Germ-free mice

The haired mice showed no signs of infection throughout the experiment. Traces of infection were seen on 3 of the 4 nu/nu mice, these consisted mainly of tiny, barely visible nodules at the infected sites. A lesion which resembled a bite mark appeared on one of the nu/nu mice at 7 days after infection. The nu/nu-bg/bg mice had more severe infections with crusts visible over most of the infected site in 2 of the 3 mice. In this group also, a lesion which looked like a bite mark appeared on one of the mice at 7 days. These marks may have been the result of self-trauma due to irritation of the infected area. No other bite marks were seen on the mice and therefore the accidental appearance, at the infected sites, of marks due to fighting is unlikely.

At days 3 and 7 after infection the scores for the nu/nu-bg/bg mice were significantly higher ($p < 0.01$ and $p < 0.05$) than those of the nu/nu mice (fig. 2).

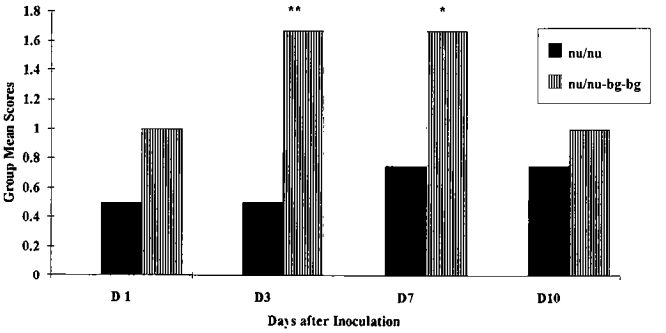

*Figure 2 : The lesion scores for germ-free normal and immunodeficient mice. Axes as figure 1. No lesions were seen on the haired (nu+) group throughout the experimental period. Lesions on the nude-beige mice (nu/nu-bg/bg) were more pronounced than those on the nude (nu/nu) mice. (**= $p < 0.01$ *= $p < 0.05$).*

Histopathology

Large differences were seen in the structure of the hair follicles of the nude and haired mice, reflecting their grossly different appearance. The follicles of the nude mice were convoluted with truncated hair shafts and often contained "keratin pearls". The epidermal layer tended to be slightly thicker in the nude mice. The uninoculated nude mice also tended to have greater numbers of mast cells in the dermis when compared with the uninoculated haired mice.

No differences attributable to the bacteriological status of the mice, i.e. gnotobiotic or germ-free, could be seen.

Very few histology specimens showed clinically apparent lesions, exceptions being the large crusted lesion on one of the nu/nu-bg/bg-xid mice, the ulcer-like lesions on one of the BALB/c nu/nu mice and the small localized "bite marks" on the nu/nu and nu/nu-bg/bg germ-free mice. However, many of the samples without visible lesions showed signs of localized inflammatory changes. Acanthosis and hyperkeratosis were the most common signs of past or continuing inflammation but only in rare instances were these areas associated with an underlying cellular infiltrate. This infiltrate was either of mast cells or, less commonly, of neutrophils. Greater numbers of dermal blood vessels seen in association with the hyperplastic areas were further evidence of inflammatory change.

Mast cells appeared to be present in the dermis of inoculated mice, of all types, in greater numbers when compared with specimens from uninoculated controls. Occasionally, clusters of mast cells could be seen directly underneath the areas of epidermal hyperplasia.

The histological examination of the large, crusted lesion (photo 3) revealed a healing scab underrun by new epidermal growth. Extensive areas of hyperplasia and dermal thickening were seen at the margins of the lesion. Neutrophils could be seen emerging from nearby blood vessels and migrating through the hyperplastic epidermis in regions where the new *stratum corneum* was not yet complete. The bulk of the lesion consisted of a mass of keratin layers interleaved with neutrophil debris. Within this structure the remnants of hair follicles were delineated by the filaments of *D. congolensis* growing within them.

The ulcer-like lesions seen in the BALB/c-nu/nu mice were associated with the presence of Gram-positive cocci, mostly single but also found in pairs or small clusters. The lesion itself consisted of a mass of neutrophil debris with scanty keratin. The epidermis beneath most of the mass of neutrophil debris was all but absent and the lesion extended into the underlying fatty tissue. Neutrophils could be seen migrating from the dermis, through the remains of the epidermis, into the infected area. At the edges of the "ulcer", parakeratosis was evident. The "bite mark" lesions on the germ-free mice were similar to the "ulcer" lesion in the absence of *D. congolensis* in Gram stained sections and the lack of involvement of hair follicles. In both lesions, the epidermis at the margins was hyperplastic but there was no regrowth of fresh epidermis underneath the main part of the lesion. Both types of lesion involved considerable neutrophil infiltration but this was the only point of similarity with the lesion on the nu/nu-bg/bg-xid mouse, which had clearly been caused by *D. congolensis*.

DISCUSSION

In this study, the absence of T cell mediated immunity in the nude mice seemed to have little influence on susceptibility to *Dermatophilus* infection. This is surprising in the

light of other reports which mention the increased susceptibility of nude mice to other infections such as fungi and yeasts (4, 8, 15). One possible explanation may lie in the idea of T cell mediated specific immunity as a back-up for the faster system of neutrophil influx in response to cytokine release from damaged epidermal cells. Infection with *D. congolensis* in the athymic mice appeared to have been successfullly contained by non-specific immune responses and perhaps the T cell response was not needed. However, the observation of a delayed, although very minor, infection on one of the nude mice suggests that a subclinical infection had occurred and had then become apparent before being dealt with, probably by the aforementioned non-specific immune defences.

There is evidence that athymic mice may have some residual gamma-delta T cell activity. Epidermal cells bearing the T cell marker Thy1 are found in nude mice. Unlike the $Thy1^+$ dendritic cells found in the epidermis of normal mice, these cells have limited expression of the genes coding for the $\gamma\delta$ T cell receptor. They also have other functional and structural differences which suggest that they are at a very early stage of differentiation. However, in spite of this, they are able to respond to low doses of interleukin-2 (12) and these cells may be able to play a role, albeit an inefficient and limited one, in the non-specific, protective response of the skin to *D. congolensis*.

In contrast, the beige gene seemed to be the factor which most influenced susceptibility to experimental infection with *D. congolensis*. The beige mutation is found in several species and corresponds to the human immunodeficiency disease Chediak-Higashi syndrome. There are several functional immune abnormalities resulting from the beige mutation in mice and it is uncertain which of them was the cause of the increased lesion severity seen in these mice. The underlying problem in beige mice appears to be a defect affecting the formation and morphology of granular cell organelles, including lysosymes, melanosomes, mast cell granules and platelet storage granules. In some of these cases the presence of characteristic "giant" granules is accompanied by functional deficiencies. The neutrophils of beige mice have reduced chemotactic and bactericidal capacity but their macrophages have been demonstrated to have a normal capacity to secrete lysosymal enzymes (reviewed in (2)). Histological study of the lesion seen on a nu/nu-bg/bg-xid mouse confirmed that neutrophils were still the predominant cells infiltrating the site of infection. The establishment of infection in spite of the influx of neutrophils suggests that they may have been inefficient at clearing the pathogen from the inoculated site.

Another important immune deficiency in these mice is the defect in endogenous NK cell activity which has led to their use in cancer research (6). An ineffective NK cell response could have been another factor in the establishment of severe lesions in the mice carrying the beige gene. Nothing is known of the role of NK cells in the immune response to *D. congolensis* and this area may be a fertile one for investigation. The appearance of the healing lesion also highlighted the importance of the growth of new epidermal tissue forming a protective wall between the infected tissue and the dermis.

The presence of the x-linked immunodeficiency gene did not increase the susceptibility of beige-nude mice to *D. congolensis*. The xid mutation causes a deficiency of T-independent B cell responses, with a B cell population that has the appearance of an immature phenotype (16) and the absence of the unusual B cell subset which carries the T cell marker, CD5 (5). $CD5^+$ B cells are found mainly in the peritoneal cavity and appear to be a separate lineage from the majority of B cells (11). In the context of immunity to *D. congolensis* these cells may not play an important part and thus explain the failure of the xid mutation to influence the outcome of infection.

The presence of commensal skin flora, such as *Bacillus* and *Staphylococcus* sp. has been shown to inhibit the growth of *D. congolensis in vitro* (9, 13). However, the lack of skin flora of the germ-free mice did not appear to affect the course of the lesions induced by experimental *D. congolensis* infection. Thus suggesting that, in this case, immune defences were more important than inhibitory bacteria. Competitive studies will be needed to further elucidate the situation, especially as the *D. congolensis* inoculum titres could not be equalized in this set of experiments. The infective dose was much heavier in the 1st experiment and may have overcome any resistance offered by the gnotobiotic skin flora.

The histopathological evidence of slight inflammation suggests that subclinical reaction to *Dermatophilus* was widespread. The focal nature of the inflammatory response also suggests that it was not due to any reaction against the inoculation process but may have indicated a subclinical infection of the skin. This may have been the case seen with one of the nude mice in experiment 1, where hair was lost from apparently normal skin and small nodules then developed at the hairless sites. The presence of *D. congolensis* in the gut of the germ-free mice also suggests that the bacterium is able to survive in previously unsuspected sites, which may help to explain its recurrence in apparently unaffected animals.

This work has raised interesting questions about the role of specific, T cell-mediated immunity in protection against *D. congolensis*. Increasingly, immunology is being led back to its roots and the importance of its less glamorous components, such as non-specific inflammation is being realized.

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Gnotobiotic mice with congenital immune deficiencies were infected with the skin pathogen *Dermatophilus congolensis*. Athymic (nude) mice with T cell deficiency were less susceptible than nude mice which also carried the beige mutation (beige-nude) with NK cell and granulocyte defects, as part of the murine equivalent of Chediak-Higashi syndrome. The additional presence of the x-linked immunodeficiency gene in other beige mutant mice, giving reduced B cell responsiveness, did not increase their susceptibility. BALB/c mice with the nude mutation and evidence of macrophage insufficiency, had a moderate level of susceptibility, greater than that of outbred nude mice but less than that of beige, nude mice. The appearance of the lesions on the haired mice was different from that on those with hairless skin (nude and beige-nude). On the haired mice thin crusts developed and healed rapidly, while on the hairless mice the lesions started as nodules and later progressed to crusts. The nude BALB/c mice developed atypical lesions, which resembled ulcers. Germ-free nude and beige-nude mice showed the same types and time course of infection as the gnotobiotic animals, suggesting that bacterial interference, by a limited skin flora, did not play a major role in defence against *D. congolensis*. However, bacteriological analysis indicated that *D. congolensis* could survive in the gut of germ-free mice. This work emphasizes the importance of non-specific immune mechanisms, such as epidermal hyperproliferation and the neutrophil, in resistance to *D. congolensis*.

Key words : Mice - Dermatophilosis - *Dermatophilus congolensis* - Experimental infection - Immunodeficiency - Lesion - Dermatology - Gene - Disease resistance.

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Se infectaron ratones gnotobióticos inmunodeficientes con *Dermatophilus congolensis*, patógenos de la piel. Los ratones desnudos (sin timo) con deficiencia de células T, fueron menos susceptibles que los ratones desnudos portadores de una mutación "beige" ("beige"-desnudos), con células NK y defectos granulocitarios, como parte de un equivalente murínico del sindrome de Chediak-Higashi. La presencia adicional del gen de inmunodeficiencia x-de unión en otros ratones "beige-mutantes", causante de una disminución en la respuesta de células B, no aumentó la susceptibilidad. Ratones BALB/c desnudos y con insuficiencia de macrófagos, presentaron un nivel moderado de susceptibilidad, mayor que aquel de los ratones desnudos, pero menor que los mutantes "beige"-desnudos. La aparición de las lesiones en los ratones con pelo fue diferente que en aquellos sin pelo (tanto desnudos como "beige"-desnudos). En los primeros, se desarrollaron finas costras que sanaron rapidamente, mientras que en los segundos, las lesiones se iniciaron como nódulos y evolucionaron luego hacia costras. Los ratones BALB/c desnudos desarrollaron lesiones atípicas, semejantes a úlceras. Los ratones desnudos libres de gérmenes y los "beige"-desnudos mostraron el mismo tipo y curso de infección que los animales gnotobióticos, sugiriendo que una interferencia bacteriana, mediante una flora dérmica limitada, no juega un papel importante en la defensa contra *Dermatophilus congolensis*. El analísis bacteriológico indica que *D. congolensis* sobrevive en el intestino de ratones libres de gérmenes. Este trabajo da énfasis a la importancia de los mecanismos de inmunidad no específica, como la hiperproliferación epidérmica y los neutrófilos, en la resistencia a *D. congolensis*.

Palabras claves : Ratón - Dermatofilosis - *Dermatophilus congolensis* - Infección experimental - Inmunodeficiencia - Lesión - Dermatología - Gen - Resistencia a la enfermedades.