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SPLEEN CELL PROLIFERATION DURING AND AFTER SKIN MYIASIS BY HUMAN BOT FLY *Dermatobia hominis*

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SUMMARY

Spleen cells from mice were examined at 5, 10, 15, 20 and 25 days post-infection (dpi) with *Dermatobia hominis* larva and at 5, 10, 15, 30 and 60 days post-larval emergence (dple). Cell proliferation *in vitro* assays were carried out with RPMI-1640 medium and larval secretory product (LSP) of *D. hominis* at 5, 10, 15, 20 and 25 days. When each group of mice was tested against each medium, significance was only seen for 25 dpi, with increasing order: LSP-10 d, -25 d, -5 d, -20 d, -15 d and RPMI. Significant results were also observed when each medium was tested against mice at each dpi or dple. Each dple group vs. each medium produced significant results only for 10 dple, with increasing order: LSP-5 d, -20 d, -25 d, -10 d, -15 d and RPMI. Comparative tests were also carried out between groups to refine certain observations. The LSPs were also analyzed using SDS-PAGE. The results prove that myiasis caused depletion of spleen cells, particularly under the effect of the LSP-10 and -15, but the cells tended to increase up to 60 dple. This *in vitro* assay may represent the real systemic immune response in the relationship LSP-*D. hominis*-host.

KEYWORDS: *Dermatobia hominis*; Mice; Skin myiasis; Spleen cell; Blastogenesis.

INTRODUCTION

Cutaneous obligatory myiasis by the human bot fly *Dermatobia hominis* (L. Jr.) larva is a Neotropical zoonotic disease that produces severe economic losses to livestock production and also has public health importance[11]. The developmental cycle of *D. hominis* is unusual in that adults are aphagous, mated females capturing and depositing their eggs on several species of flies, including *Musca domestica* and *Stomoxys calcitrans*. After about a week, when the phoretic host feeds or lands on a mammalian host, the bot fly larvae hatch and immediately penetrate the animal's skin. Although the pathological and immunological aspects of human bot fly myiasis have been little studied under field conditions[6,26], mice have proved to be a suitable laboratory model for the relationship between *D. hominis* and its hosts[5,13,18,21]. The response of mouse spleen cells during and after infection by larvae of *D. hominis* is described here.

MATERIAL AND METHODS

Animal infection: Each animal from 10 groups of six male Swiss mice (20-25g) was submitted to skin infection with one newly hatched larva of *D. hominis* reared in our laboratory[9]. The mice were sacrificed and spleens obtained from groups 1-5 at 5, 10, 15, 20 and 25 days-post-infection (dpi), respectively. Spleens from groups 6-10 were collected at 5, 10, 15, 30 and 60 days post-larval-emergence (dple) respectively. Spleens from corresponding negative control 11th group (without infection = 0 dpi) were also examined.

Larval secretory product (LSP): Five groups with five male adult Wistar rats (190-220g) were infested with four newly hatched larvae of *D. hominis* and sacrificed at 5, 10, 15, 20 and 25 dpi to collect the larvae from the skin tissues[9]. Larvae at each infested date were washed three times with RPMI-1640 medium (Sigma) and conditioned in Falcon tubes with 2 mL of RPMI for incubation at 37 ºC in a 5% CO_2 atmosphere for 24 h. Crude *D. hominis* LSP was obtained at 5 d (LSP-5d) from 30 first larvae (L_1); at 10 d (LSP-10 d) or 15 d (LSP-15 d) from 15 second larvae (L_2) and at 20 d (LSP-20 d) or 25 d (LSP-25 d) from 10 third larvae (L_3). The LSP of each different days or larval instars was stored in liquid nitrogen until ready for use.

Concentration and electrophoreses of LSP: Protein concentration of the LSP was determined using the Bradford (1976) technique and subjected to continuous electrophoresis using 10% SDS polyacrylamide gels, under reducing conditions[14]. The protein concentration used to electrophoresis was of 50 µg/LPG µL. The separated proteins were stained with Coomassie blue.

In vitro spleen proliferation response: Spleens of each group (1-11) rats were washed three times with RPMI, macerated, immersed in 20 mL RPMI, filtered and the filtrate centrifuged at 1400 RPM for 10 min.

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Erythrocytes were lysed by re-suspending the pellet in 300 µL Tris-ammonium chlorite plus 30 mL MEM (minimum essential medium eagle, Sigma) and washing with the same solution. The spleen cells were suspended in 20 mL of RPMI, diluted in 10 mL Tweck solution and counted under a Neubauer camera. Cell proliferation assays (in triplicate) were done in response to RPMI and LSP at 5 d, 10 d, 15 d, 20 d and 25 d. Spleen cells ($1x10^6$) were cultured in 200 µL culture medium in 96-well flat-bottom plates. Except for the control group, 25 µg/mL of LSP was introduced in the culture medium in each plate and incubated at 37 ºC in a CO_2 incubator for 5 d. At 18 h before the final incubation, 0.5 µCi of titrated thymidine (specific activity, 37 Ci/mL; New England Nuclear, Boston, MA, USA) was added to each well. Cells were harvested onto glass fiber paper and the incorporated radioactivity was measured by a liquid scintillation β-counter. The results were expressed as mean ± S.E. of c.p.m. values.

Statistical analyses: The results of the blastogenesis were analyzed by non-parametric methods (GraphicPad Prism 4.03 software) by comparison among all groups (Kruskal-Wallis test) and between pairs of groups (Mann-Whitney U test) using independent variables. The data are represented in box and whisker plots, the middle line corresponding to the median and the lower and upper line representing the 25 and 75 percentiles, respectively (50% of the data fall within the box, and 25% each above the below). The whiskers above and below the box plot indicate 95% and 5% percentiles, respectively. Values were considered significant at $p < 0.05$.

RESULTS

Animal infection: The L_1 of *D. hominis* changed to L_2 at six dpi, the L_3 moulted at 16 dpi and the mature warbles escaped from the host around 30 dpi. A scar formed on the skin of each mouse 7-10 days later. In the laboratory L_3 left the experimentally infested mice and pupated, resulting in fertile adult *D. hominis*.

In vitro spleen cell proliferation from infested mice: When the response of each group (1-5) with each substrate was analyzed, significance was only seen at 25 days of infection (group 5) increasing as follows: LSP-10 d, -25 d, -5 d, -20 d, -15 d and RPMI (Fig. 1). In the reciprocal analysis, *i.e.,* of each substrate with each mouse group, significance was observed in all assays, highest values being seen for RPMI (Fig. 2): 25, 5, 20, 10, 15 dpi and control; LSP-5 d (Fig. 3): 25, 5, 10, 20, 15 dpi and control; LSP-10 d (Fig. 4): 25, 10, 5, 20, 15 dpi and control; LSP-15 d (Fig. 5): 25, 5, 10, 20, 15 dpi and control; LSP-20 d (Fig. 6): 25, 10, 5, 15 dpi, control and 20 dpi; LSP-25 d (Fig. 7): 25, 10 dpi, control, 5, 20 and 15 dpi. The results of between-group analyses (Mann-Whitney U test) are summarized in Table 1.

In vitro spleen cell proliferation from mice at days-post-larval-emergence: Analyses of each mouse group *vs* each medium were only significant for 10 dple, values increasing in the following order: LSP-5 d, -20 d, -25 d, -10 d, -15 d and RPMI. Significant results were only obtained for three assays, when each medium was analyzed with each mouse group, values increasing as follows: to RPMI: 30, 10, 15, 60, 5 dple and control; LSP-10 d: 30, 10, 15, 5, 60 dple and control and LSP-15 d: 30, 10, 15 dple, control, 5 and 60 dple. Additional test results are also shown in Table 2.

Concentration and electrophoreses of LSP: Separation of LSP molecules by SDS-PAGE revealed multiple protein bands (Fig. 8), their molecular weights ranging from 12-50 kDa. Major stained protein bands of 12-25 kDa were seen in all larval stages.

DISCUSSION

The metamorphosis of *D. hominis* inside mammalian skin occurs below the point of L_1 penetration, in deep dermis[21]. This contrasts with other oestrid species, *e.g.* rodent bot fly (*Cuterebra*) and cattle bot fly (*Hypoderma*) where the L_1 migrate within the host skin before the second and third instars fix in the cutaneous tissues to form the mature warble[16,24,25]. The multi-host parasitism exhibited by *D. hominis* also differs from the host specificity seen in other bot flies. There is usually a balance between the host and the three larval stages[6]. Preliminary studies

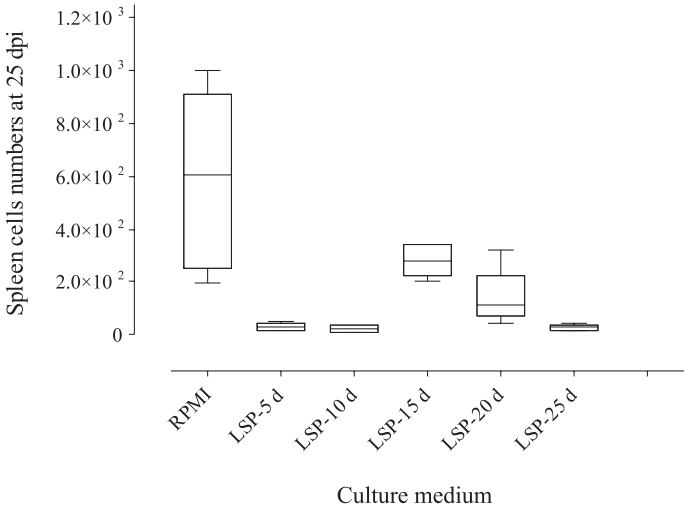

Fig. 1 - Box and whisker plots representing the numbers of spleen cells from mice at 25 days post-infection (dpi) by *Dermatobia hominis* submitted to RPMI and larval-secretory-product (LSP).

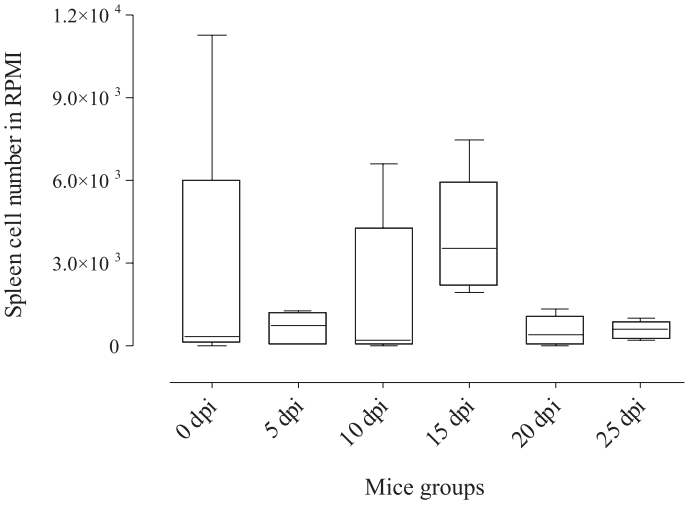

Fig. 2 - Box and whisker plots representing the numbers of spleen cells from mice control and at days post-infection (dpi) by *Dermatobia hominis* submitted to RPMI.

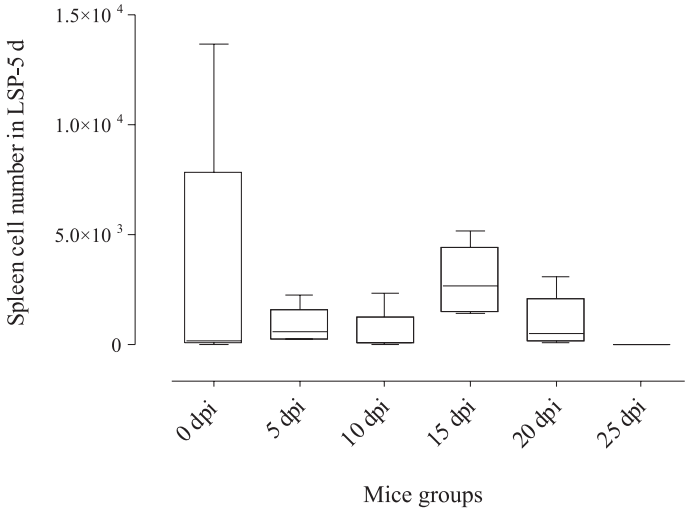

Fig. 3 - Box and whisker plots representing the numbers of spleen cells from mice control and at days post-infection (dpi) by *Dermatobia hominis* submitted to larval-secretory-product (LSP) at five days.

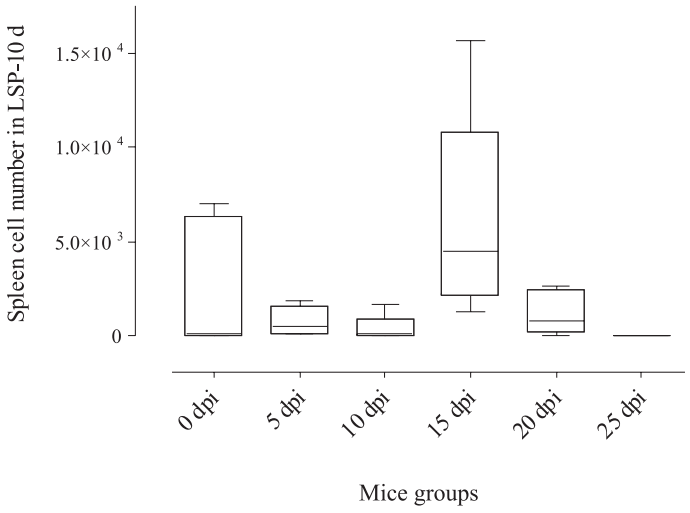

Fig. 4 - Box and whisker plots representing the numbers of spleen cells from mice control and at days post-infection (dpi) by *Dermatobia hominis* submitted to larval-secretory-product (LSP) at 10 days.

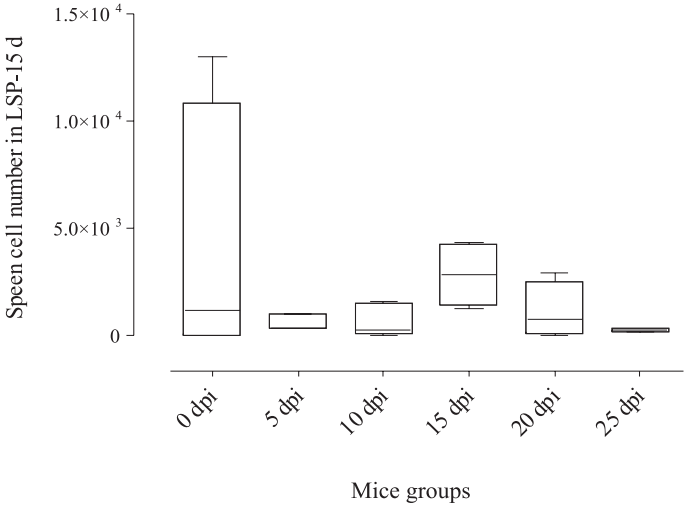

Fig. 5 - Box and whisker plots representing the numbers of spleen cells from mice at days post-infection (dpi) by *Dermatobia hominis* submitted to larval-secretory-product (LSP) at 15 days (by Kruskal-Wallis test).

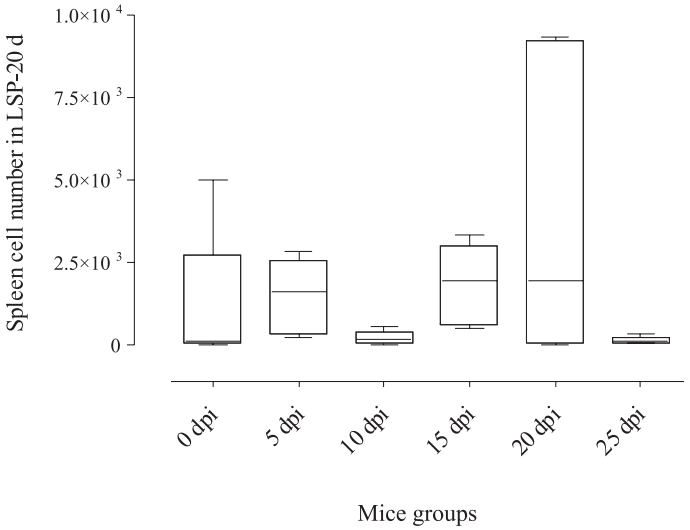

Fig. 6 - Box and whisker plots representing the numbers of spleen cells from mice at days post-infection (dpi) by *Dermatobia hominis* submitted to larval-secretory-product (LSP) at 20 days.

have demonstrated that bot burden, instar type, infection or re-infection can regulate the host's inflammatory process as well as its humoral and cellular immune responses[4,6,7,17,20].

As soon as *D. hominis* L_1 invades the host's skin, cellular and molecular changes occur in response to the mechanical presence of the larval stage as well as its secretory products. A chronological recruitment of the cells to skin lesions has been studied during and after infection in rats[21,22]. Cellular or humoral responses against human bot fly myiasis using rabbits, cattle and mice were recently re-examined[17]. However, cytological tissue changes (or damage) in warble-infested skin has been poorly studied under natural or controlled conditions. Circulating leucocytes in hosts with *D. hominis* myiasis are reported in cattle[1,8] and rats[10]. Except for observations of alterations in response to migration of the L_1 of *Cuterebra* and *Hypoderma*, there have been few studies of the host cellular pathways in skin myiasis[6,26].

Oestrid LSP has been most studied in *H. lineaum* L_1 midgut, from which enzymes (hypodermins) with immune regulation properties were isolated. Both hypodermin (trypsins) A and B[15,28] deplete complement cytolytic activity[2,3], B also reducing leukocyte and lymphocyte blastogenesis, as well as the production of interleukin-2 and prostaglandin[19]. Although molecules from *D. hominis* LSP have not been identified, our results confirm that cellular suppression occurs in mice with human bot fly myiasis. Based on the results of studies using crude extracts of *D. hominis* L_2 and L_3, it has been suggested that serine-

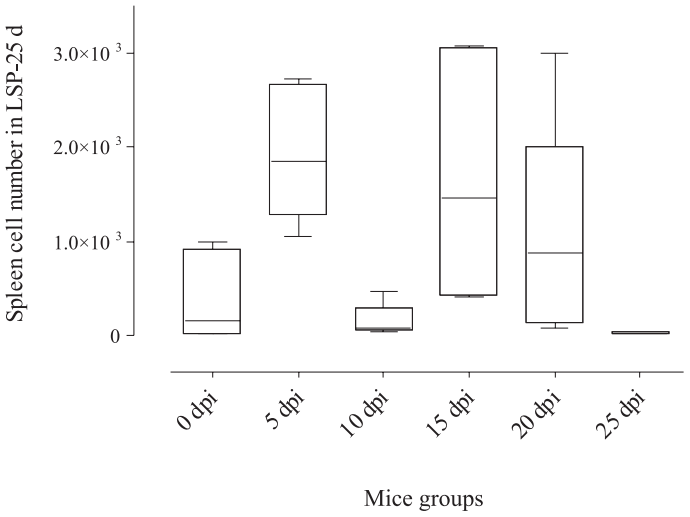

Fig. 7 - Box and whisker plots representing the numbers of spleen cells from mice at days post-infection (dpi) by *Dermatobia hominis* submitted to larval-secretory-product (LSP) at 25 days.

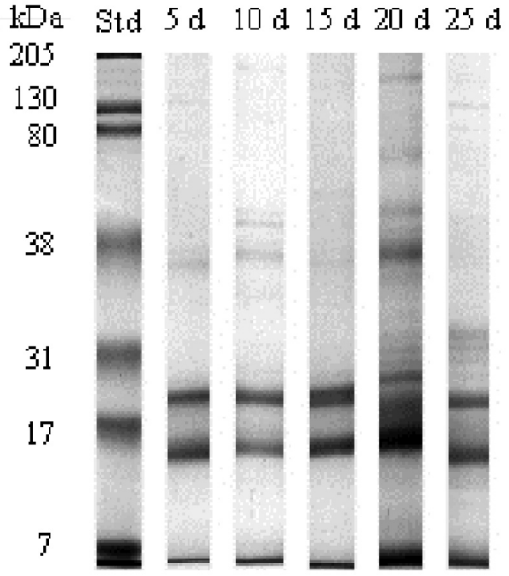

Fig. 8 - SDS-PAGE (10 %) of larval-secretory-product from *Dermatobia hominis*: at five days (first instar), 10 or 15 days (second instar) and 20 or 25 days (third instar).

proteases occur in such larvae[23]. Our data reveal a reduction in spleen cell numbers during *D. hominis* myiasis.

Assays with each dpi vs. each medium revealed that significant reductions only occurred at 25 dpi and that RPMI and LSP-15d produced most cell proliferation, followed by LSP-20 d. The immunosuppressive effect of the larvae could be switched off at 15d, when the L_2 molts to L_3. Based on the other analysis (U test), mice have more spleen cells at 15 dpi on LSP-15d than on other media, and more on LSP-10 d than LSP-20 or -25d. When the reciprocal tests were carried out, *i.e.* each

Table 1
Analyses of spleen cells from mice control and days-post-infestation (dpi) by *Dermatobia hominis* larvae in response to media (RPMI and larval secretory product = LSP) of warbles at 5, 10, 15, 20 and 25 days and each medium vs each group

Assays	Significant results by Mann-Whitney U test
Mice control (0 dpi) vs medium	No
Mice with 5 dpi vs each medium	Pro-25 dpi x RPMI, LSP-5 d, -10 d and -15 d
Mice with 10 dpi vs each medium	No
Mice with 15 dpi vs each medium	Pro-LSP-10 d x LSP-20 d, -25 d
Mice with 20 dpi vs each medium	No
Mice with 25 dpi vs each medium	Pro-RPMI x LSP-5 d, -10 d, -20 d and -25 d
	Pro-LSP-15 d x LSP-5 d, -10 d, -20 d and -25 d
	Pro-LSP-20 d x LSP-5 d, -10 d and -25 d
RPMI *vs* each mice group	Pro-15 dpi x 5 dpi, 10 dpi, 20 dpi and 25 dpi
LSP-5 d vs each mice group	Pro-control x 25 dpi
	Pro-5 dpi x 25 dpi
	Pro-10 dpi x 25 dpi
	Pro-15 dpi x 5 dpi, 10 dpi, 20 dpi and 25 dpi
	Pro-20 dpi x 25 dpi
LSP-10 d vs each mice group	Pro-control x 25 dpi
	Pro-5 dpi x 25 dpi
	Pro-10 dpi x 25 dpi
	Pro-15 dpi x 5 dpi, 10 dpi, 20 dpi and 25 dpi
	Pro-20 dpi x 25 dpi
LSP-15 d vs each mice group	Pro-5 dpi x 25 dpi
	Pro-15 dpi x 5 dpi, 10 dpi and 25 dpi
LSP-20 d vs each mice group	Pro-5 dpi x 10 dpi and 25 dpi
	Pro-15 dpi x 10 dpi and 25 dpi
LSP-25 d vs each mice group	Pro-5 dpi x control, 10 dpi and 25 dpi
	Pro-10 dpi x 25 dpi
	Pro-15 dpi x control, 10 dpi and 25 dpi
	Pro-20 dpi x 25 dpi

Pro = favorable

Table 2
Analyses of spleen cells from mice control and days-post-larval-emergence (dple) of *Dermatobia hominis* in response to media (RPMI and larval secretory product = LSP) of warbles at 5, 10, 15, 20 and 25 days, and each medium versus each group

Assays	Significant results by Mann-Whitney U test
Mice with 5 dple vs each medium	No
Mice with 10 dple vs each medium	Pro-RPMI x 5 d, 10 d, 20 d and 25 d
	Pro-10d x 20 d
	Pro-15d x 5 d, 10 d, 20 d and 25 d
Mice with 15 dple vs each medium	No
Mice with 30 dple vs each medium	No
Mice with 60 dple vs each medium	No
RPMI vs each mice group	Pro-5 dple x 30 dple
	Pro-10 dple x 30 dple
	Pro-15 dple x 30 dple
	Pro-60 dple x 10 dple, 15 dple and 30 dple
LSP-5 d vs each mice group	Pro-60 dple x 30 dple
LSP-10 d vs each mice group	Pro-control
	Pro-10 dple x 30 dple
	Pro-60 dple x 10 dple, 15 dple and 30 dple
LSP-15 d vs each mice group	Pro-10 dple x 30 dple
	Pro-60 dple x control, 5 dple, 15 dple, 30 dple
LSP-20 d vs each mice group	Pro-15 dple x 10 dple, 30 dple
	Pro-60 dple x 10 dple, 30 dple
LSP-25 d vs each mice group	Pro-10 dple x 30 dple
	Pro-60 dple x 10 dple, 15 dple, 30 dple

Pro = favorable

medium vs. each dpi, spleen cell suppression was also seen at the end of the infection. In this case more cells also found at -15 dpi, indicating similar significance with RPMI, LSP-5d and -10 d (Table 2).

Among control and dple mice vs. each medium the unique significant result at 10 dple indicates that RPMI and LSP-15 d have similar effects on spleen cell numbers, producing more cells than all the other assays. Otherwise, irrespective of the medium used vs. dple the lowest cell numbers in mice occurred at 30 dple. However, at 60 dple mice usually have more cells than in other assays. If 60 dple mice are compared with control group (uninfested mice) there is equalization between them.

Based on SDS-PAGE, two marked bands of small peptides expressed in *D. hominis* LSP revealed the presence of homologous molecules in the three parasite instars. In contrast with L_1 and L_2, large amounts of peptides were observed in L_3 at 20d after infection. It is possible that at this point the L_3 displays more LSP due to voracious parasitism. This is borne out by the results of histopathological studies[21]. The LSP of *D. hominis* may have similar peptides (23 and 18 kDa) to those described in *O. ovis*: L_1, L_2 and L_3 (larval extract), LSP and salivary gland of L_3[12]. The warble fly *H. lineatum* (De Villers) has protease (trypsin-like) molecules which can break down bovine C_3[3]. The peptide of approximately 38 kDa present in L_2 and L_3 *O. ovis*[27] may also be present in *D. hominis*. A spectrum of molecules from 60-97 kDa was detected in crude extract of *D. hominis* L_2 and L_3[8]. Proteinases, probably serine-proteases of m.w. 13 and 22 kDa, were identified in crude extracts of *D. hominis* L_2 and L_3, and an unidentified molecule of 50 kDa also seen in both instars[23]. Further studies of *D. hominis* LSP are in progress in our laboratory, which should provide new information on human bot fly myiasis.

RESUMO

Proliferação de células do baço durante e após miíase por *Dermatobia hominis*

Células do baço de camundongos foram examinadas aos 5, 10, 20 e 25 dias pós-infecção (dpi) com *Dermatobia hominis* e examinadas aos 5, 10, 15, 30 e 60 dias pós-emergência da larva (dpel). As células foram cultivadas em meio RPMI-1640 contendo, ou não (controle), produtos de secreção das larvas (PSL) de *D. hominis* com idade de 5, 10, 15, 20 e 25 dias. Em cada grupo com cinco camundongos testados nos meios de cultura, o número de células foi significativo para 25 dpi, com crescente aumento na seguinte ordem: PSL-10 d, -25 d, -5 d, -20 d, -15 d e RPMI. Resultados significantes foram também observados nos testes entre cada meio contendo células tanto de camundongos dpi ou dpel. Em cada dpel grupo versus meio significância foi somente verificada para 10 dpel, na ordem crescente: PSL-5 d, -20 d, -25 d, -10 d, -15 d e RPMI. Testes comparativos foram também realizados entre grupos. O PSL foi analisado sob SDS-PAGE. Os resultados provam que a miíase causou depleção de células do baço, particularmente sob efeito do PSL-10 e -15, mas ocorreu normalidade do número de células aos 60 dpel. Este ensaio *in vitro* pode representar uma resposta imune sistêmica na relação PSL-*D. hominis*-hospedeiro.

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