Mucosal inflammatory responses after experimental infection with gastrointestinal nematodes in resistant and susceptible sheep naturally infected with *Oestrus ovis*

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**SUMMARY**

Recent epidemiological observations from field studies indicated that sheep selected for resistance to digestive nematodes were more infected than susceptible ones with the nasal bot fly, *Oestrus ovis*. The present study was undertaken to verify these informations and to explore the possible associated mechanisms. After a whole grazing season and an anthelmintic drench, two groups of 6 rams belonging respectively to 2 flocks with different resistance status to parasitic nematodes were experimentally infected with a mixture of *Haemonchus contortus*, *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* infective larvae. The worm populations in the gastrointestinal tract, the *Oestrus ovis* populations in the nasal cavities and the intensity of inflammatory reactions (number of mast cells, eosinophils and globule leucocytes) in the respiratory and digestive mucosae were compared in the 2 groups. No differences were found in the worm numbers and in the cell populations in the digestive tract and this lack of difference was due to acquisition of immunity to nematodes in both lines of rams at the end of the grazing season. In contrast, the mean number of *O. ovis* larvae was higher in the resistant group compared to the susceptible one (6.3 vs 2.8) and the number of eosinophils was significantly higher in the septum and ethmoid in this resistant group. These results tend to confirm the higher susceptibility to *O. ovis* in animals selected for resistance to digestive nematodes. They also suggest that the intensity of inflammation in the nasal mucosa might be positively related to the intensity of infection with *O. ovis*.

**KEY-WORDS** : trichostrongyles - *Oestrus ovis* - genetic resistance - sheep - inflammatory response

**RéSUMÉ**

Réponse inflammatoire des muqueuses lors d’infestations par des trichostrongyles et par *Oestrus ovis* chez les moutons de 2 lignées sélectionnés pour leur divergence de réponse (résistants ou sensibles) vis-à-vis des nématodes du tube digestif. Par H.T. YACOB, H. HOSTE, Ph. JACQUIET, C. DURANTON GRISEZ, Ph. DORCHIES et L. GRUNER.

Des données épidémiologiques récentes, acquises en conditions naturelles, suggèrent que des moutons sélectionnés pour leur résistance vis-à-vis des nématodes du tube digestif seraient par contre plus sensibles aux infestations par *Oestrus ovis*. Cette étude a été mise en œuvre pour vérifier ces observations en conditions expérimentales et explorer les mécanismes en cause. A la suite d’une saison de pâturage, 2 lots de 6 béliers appartenant à 2 lignées sélectionnées pour la divergence de leur réponse aux nématodes parasitaires du tractus digestif ont été traités par un anthelminthique puis infestés expérimentalement par un mélange de larves infestantes d'Haemonchus contortus, Trichostrongylus colubriformis et Teladorsagia circumcincta. Les populations de vers dans le tractus gastro-intestinal, le nombre d’*Oestrus ovis* dans les cavités nasales et l’intensité de la réponse inflammatoire (nombre de mastocytes, de globule leucocytes et de polynucléaires éosinophiles) dans les muqueuses respiratoires et digestives ont été comparés dans les 2 groupes. Malgré leur appartenance à des lignées différentes, aucune différence n’a été détectée dans les populations de vers et dans les réactions cellulaires des muqueuses digestives. Cette absence de différences a été associée à l’acquisition d’une immunité face aux trichostrongyles dans les 2 lots d’animaux à la fin de la saison de pâturage. A l’inverse, chez les moutons résistants, le nombre moyen de larves d’*O. ovis* était plus élevé que chez les moutons sensibles (6.3 vs 2.8) et le nombre de polynucléaires éosinophiles était significativement augmenté dans le septum et l’ethmoïde. Ces résultats viennent confirmer la plus forte sensibilité à *O. ovis* des ovins sélectionnés pour leur résistance génétique face aux nématodes digestifs. Ils suggèrent aussi que l’intensité de l’inflammation dans les muqueuses nasales pourrait être positivement liée à l’intensité des infestations par *O. ovis*.

**MOTS-CLÉS** : nématodes trichostrongyles - *Oestrus ovis* - résistance génétique - ovins - réponse inflammatoire des muqueuses.
1. Introduction

The increasing development of resistances to anthelmintics in nematode populations, which parasite the digestive tract of ruminants imposes the seek for alternative solutions of control. Among the available options, genetic resistance to gastrointestinal nematodes in sheep is well documented, and resistance, measured by egg excretion, is an heritable trait used in selection of resistant or susceptible lines which have been set up, mainly in Australia and New Zealand [7, 16, 23].

In field conditions, sheep and goats are infected with various species of endo- or ectoparasites. Therefore, one of the key issues which has to be considered by the different programs for genetic selection is to make sure that the selection for resistance to gastrointestinal nematodes is not associated with counter selection for higher susceptibility to other pathogens, including some parasites. In Australia, the lines selected for resistance to one nematode species after experimental infections were experienced to other species to determine the extent of resistance [21, 22]. In natural multispecific infection, BISSET et al. [2] and GRUNER et al. [9, 10] found that the worm burden of nearly all nematode species was decreased in lambs issued from resistant rams. In the two cases, resistant sheep were protected to the main trichostrongyle species, with some specific variations. On the other hand, in France, it has been shown that Merinos of Arles ewes were more resistant after natural infection with trichostrongyles than Romanov ones but, in contrast, were more infected with *Fasciola hepatica* [8]. Similarly, observations from an epidemiological experiment conducted for 3 years on two groups of sheep divergent by their resistance status to parasitic nematodes indicated that higher resistance to trichostrongyles was repeatedly associated with higher number of *Oestrus ovis* in the upper respiratory system [12]. In addition, histological examination of the mucosae from the nasal cavities and digestive tract showed that these differences in parasite populations were related to divergence in the intensity/extent of the inflammatory processes. Nevertheless, these information were acquired only following natural infections. No data were available on the response of these divergent lines of sheep to experimental infections with nematodes as well as on possible consequences on the *O. ovis* populations. These 2 points were the main objectives of the current study concurrently with the observations of the associated mucosal response in the digestive tract and nasal cavities in order to explore the possible mechanisms involved.

2. Materials and methods

A) EXPERIMENTAL DESIGN

The study was conducted on 12 rams which came from two flocks differing by their status for genetic resistance to nematode infections. Six animals belong to a selected resistant flock to *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* and the six other ones to a susceptible flock. The rams were aged from 2 to 4 years at the start of experiment. The two groups have been grazing two neighbouring pastures for 8 months from April to November 1999 before the beginning of experiment. During grazing, the rams were naturally infected by *Oestrus ovis* and three different trichostrongyle species, *Teladorsagia circumcincta, Trichostrongylus colubriformis* and *Haemonchus contortus*. The mean faecal egg counts during October-November 1999 (end of the grazing season) in the resistant and susceptible groups ranged respectively from 215 to 810 and 1575 to 4890 eggs per gram (see table I). The rams were then removed from pastures, housed and treated with a broad-spectrum anthelmintic (fenbendazole at the recommended ovine dose, Hoechst Roussel Ltd.) to remove the worm populations burdens but not the larval populations of *O. ovis* acquired outdoors. One month after treatment, both groups were experimentally challenged with 15 000 infective larvae composed equally with each of the three following species: *Trichostrongylus colubriformis, Haemonchus contortus* and *Teladorsagia circumcincta*. Five weeks after the challenge, all the animals were killed, and opened along the abdominal mid-line. The abomasum and small intestines of each animal were processed for counts in the organ contents and washings. The stage of development of the worms recovered were determined. In addition, the heads of the ewes were split opened and the number and stages of *O. ovis* were determined according to the method previously described [24].

B) HISTOLOGICAL SAMPLES

For counting of mast cells, eosinophils and globule leucocytes, tissue samples were taken from three anatomical regions in the upper respiratory tract: i.e. the septum, turbinates, and ethmoid and from three sites of the digestive tract: i.e. pylorus, fundus and small intestine. From each anatomical region, two tissue samples of one square centimeter in size were sectioned side to side and were fixed respectively either in 10 % buffered formaldehyde or Carnoy’s fixative. Further, the tissue samples were included in paraffin wax and tissue sections of 5 µm were performed, mounted on slides using glycerinated albumin (BDH), then dried for 12 hours in an incubator at 40°C. From each experimental animal and each anatomical site, six histological slides were prepared. The tissue samples fixed with formalin were stained with Haematoxylin-Eosin to count globule leucocytes and eosinophils and the tissues fixed with Carnoy’s were stained with Alcian Blue /Safranin for mast cells.

Globule leucocytes, mast cells and eosinophils were counted in the mucosae under a x400 magnification using a 10 x 10 calibrated graticule. Ten fields, randomly selected, were observed per animal for each histological region.

C) STATISTICAL ANALYSIS

Comparison of the results obtained from cell counting in the resistant and the susceptible group was performed using the non-parametrical test of Mann and Whitney.
Results

A) PARASITE POPULATIONS

The nematode and *O. ovis* populations recovered at necropsy in each experimental group are summarized in Table I. The resistant sheep were infected on average with a total of 596 worms, while the susceptible ones with 574 worms. These numbers correspond with very low levels of larval establishment, i.e., nil for 11 out of 12 rams for *T. colubriformis* and less than 10% for *H. contortus*. Whatever the nematode species, no significant differences were found in the number of worms between the resistant and the susceptible group.

For *O. ovis* populations recovered from the nasal cavities, the total number of larvae in the group of sheep resistant to nematodes (6.3 L1) was more than twice the number of larvae counted in the susceptible group (2.8 L1). Nevertheless, the difference was non significant.

B) CELL POPULATIONS IN THE MUCOSAE OF THE GASTROINTESTINAL AND UPPER RESPIRATORY TRACTS (Fig. 1 and 2)

In the gastrointestinal tract, no significant differences were found between the resistant and the susceptible rams, irrespective of the cell type and the histological section. In contrast, in the nasal cavities, higher number of eosinophils (in the connective tissue and the epithelium) and, to a lesser extent, of globule leucocytes, were observed in the 3 anatomical sites (septum, turbinates and ethmoid) in the sheep from the resistant group to nematodes. These differences in inflammatory cell populations were statistically significant between both experimental groups for the mucosal and the epithelial eosinophils in the septum and for the mucosal eosinophils from the ethmoid.

4. Discussion

The objectives of this work were 1) to examine the difference in response to an experimental mixed challenge infection with trichostrongyles in two groups of sheep previously selected for divergent status in regard of resistance to natural infections; 2) to evaluate possible consequences of this challenge infection on established population of *Oestrus ovis* acquired in natural conditions; 3) to explore the possible mechanisms involved both in the digestive and in the upper respiratory tract.

The animals from the two groups were issued from two flocks selected for resistance or susceptibility to nematodes for several years (2 to 4 years) [11]. Previous parasitological data from field studies have indicated that the two flocks showed clear divergence in the intensity of parasite infections,

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Table I. — Number of nasal bot fly larvae and nematodes, 35 days after challenge with 5000 L3 of each of the 3 worm species. Comparison of the parasite populations between two groups of rams belonging to flocks selected for resistance or susceptibility to nematodes, as indicated by the mean egg excretion (EPG) from 3 repeated samples at the end of grazing season, before the start of the experiment.

<table>
<thead>
<tr>
<th>No and Group</th>
<th>Mean EPG</th>
<th><em>Oestrus ovis</em></th>
<th>Nematodes</th>
<th><em>H. contortus</em></th>
<th><em>T. colubriformis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L1</td>
<td>L2</td>
<td>L3</td>
<td>circumcincta</td>
</tr>
<tr>
<td>9067 R</td>
<td>215</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>9075 R</td>
<td>243</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>8117 R</td>
<td>470</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>8129 R</td>
<td>672</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>917</td>
</tr>
<tr>
<td>9078 R</td>
<td>775</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9080 R</td>
<td>810</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td><strong>MEAN</strong></td>
<td><strong>531</strong></td>
<td><strong>6.3</strong></td>
<td><strong>0</strong></td>
<td><strong>0</strong></td>
<td><strong>172.8</strong></td>
</tr>
<tr>
<td>Establishment rate %</td>
<td>3.45</td>
<td>8.5</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| 8121 S       | 1575      | 2  | 0  | 0  | 349           | 578                 | 0                    |
| 8120 S       | 1715      | 0  | 0  | 0  | 0             | 12                  | 0                    |
| 9108 S       | 2140      | 11 | 0  | 0  | 1077          | 540                 | 0                    |
| 9094 S       | 2625      | 8  | 0  | 0  | 37            | 240                 | 0                    |
| 9100 S       | 2900      | 6  | 0  | 0  | 12            | 41                  | 220                  |
| 9105 S       | 4890      | 1  | 0  | 0  | 12            | 41                  | 220                  |
| **MEAN**     | **2641**  | **2.8** | **0** | **0** | **247.7** | **289.3** | **36.7** |
| Establishment rate % | **4.9** | **5.8** | **0.7** |

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FIGURE 1. — Mean number of eosinophils, globule leucocytes and mast cells in the abomasal and intestinal mucosae of the resistant (group R) and susceptible (group S) rams following experimental challenge.

FIGURE 2. — Mean number of eosinophils, globule leucocytes, mast cells and intraepithelial eosinophils in the mucosae of the upper respiratory tract in the resistant (group R) and susceptible (group S) rams (Statistical significance : * P < 0.05).
measured either by faecal egg excretion or by worm counts at necropsy. The repeated faecal egg count measurements during the 1999 grazing season confirmed demonstrated a significant difference between the two groups of rams used for the experiment. Lower worm burdens and establishment rates were therefore expected following the experimental infection in the resistant animals compared with their susceptible counterparts. However, the actual results did not show any statistical difference in worm populations between both groups. In addition, no statistical difference were shown in the number of inflammatory cells between the resistant and susceptible rams. It is important to note that in both experimental groups, low worm establishment rates were recorded after challenge, irrespective of the parasite species. Such low establishment rates are generally the sign of development of a strong immune response against the parasites. Because animals from both groups were adults and have been exposed to a high natural infection during autumn before the start of the experimental study, one possible explanation for the lack of difference between both groups is that the susceptible rams also acquired an efficient immunity against the nematodes. Previous works in Australia or New Zealand on genetically selected lines yet suggested that differences in responsiveness between resistant and susceptible lines are more due to a faster acquisition of immune response rather than to the presence or absence of ability to rise an effective response [7].

Whereas no difference was observed both in worm populations and in the mucosal inflammatory cells within the gastrointestinal tract, differences were assessed in the upper respiratory tract. A stronger cellular response was paradoxically associated with an increased larval burden in the sheep from the resistant line. Although the difference was not statistically significant, the number of O. ovis larvae in the resistant sheep was more than twice the larvae recovered from their susceptible counterpart. In addition, the number of intraepithelial and mucosal eosinophils and, to a lesser extent, of globule leucocytes were higher in the resistant animals, with statistical differences assessed for eosinophils in two anatomical sites.

The current results on O. ovis populations confirm previous data obtained in field conditions where higher number of O. ovis larvae were repeatedly detected in sheep from the flock, resistant to nematodes [12]. Informations on the related consequences on resistance/susceptibility to other pathogens associated with genetic selection for resistance to nematodes remain few. It has generally been recognised that selection against one nematode species improve also the resistance to the other species of the gastro intestinal tract [6, 10, 21, 22]. On the other hand, some results from epidemiological studies suggest that increased resistance to digestive nematode may be associated with a higher receptivity to other parasites [8].

The resistance to gastrointestinal trichostrongyles has been associated with a number of immunological and inflammatory factors. Among those, one of the principal mechanisms described is an increased number of mucosal mast cells and eosinophils in the digestive tract [1, 13, 14, 15, 20]. It has been proposed that genetic resistance to nematode infections correspond in fact with a selection for a higher ability to mount an inflammatory response in the mucosae, particularly in regard of type 1 hypersensitivity reactions [19]. To this respect, the more intense cellular response observed in the upper respiratory mucosae in rams from the resistant line is in agreement with this proposal, even though it occurs out from the digestive tract. On the contrary, the association of this higher inflammatory response with higher numbers of Oestrus larvae appears more surprising. Previous works have indicated that infections with O. ovis was followed by recruitment of numerous mast cells and eosinophils within mucosae of respiratory tract in contact with larvae or in areas where larval migration was taking place [3, 4, 17, 18]. However, the role of these cells in host resistance to oestrus, (to limit new infection, to induce larval death or to delay development) remains unclear. In natural infections with O. ovis, a multifactorial analysis has established a positive correlation between the number of mucosal eosinophils and the total Oestrus burden [18]. Results from our work support this previous observation and even suggest that recruitment of eosinophils are positively related with the larvae and could be favourable for establishment and for development of Oestrus. However, our results contradict in vitro observations suggesting that eosinophils reduce the life span of Oestrus larvae [5]. Further investigations are clearly needed to elucidate the exact role of mucosal inflammatory cells in the pathology of O. ovis and in the host-parasite relationship.

Monospecific parasitic infections of small ruminants are very rare in the field. Therefore, it is important to consider the various risk factors, before setting up strategies for prophylactic methods against parasites. The results of our study confirm the need to explore response to other parasites in lines of sheep selected for resistance to nematodes before practical application and integration of the resistant traits in the selection scheme may be achieved. In addition, our results raised also questions on the relationship between inflammation and Oestrus infection in sheep. They suggest that convergent effector mechanisms in the respiratory and digestive mucosae might lead to divergent effect for control of the parasite populations. Lines of responder and non responder sheep to nematodes may provide a powerful biological tool to examine this question.

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