寄生第4例. 寄生虫学雑誌, 45、 333-337.

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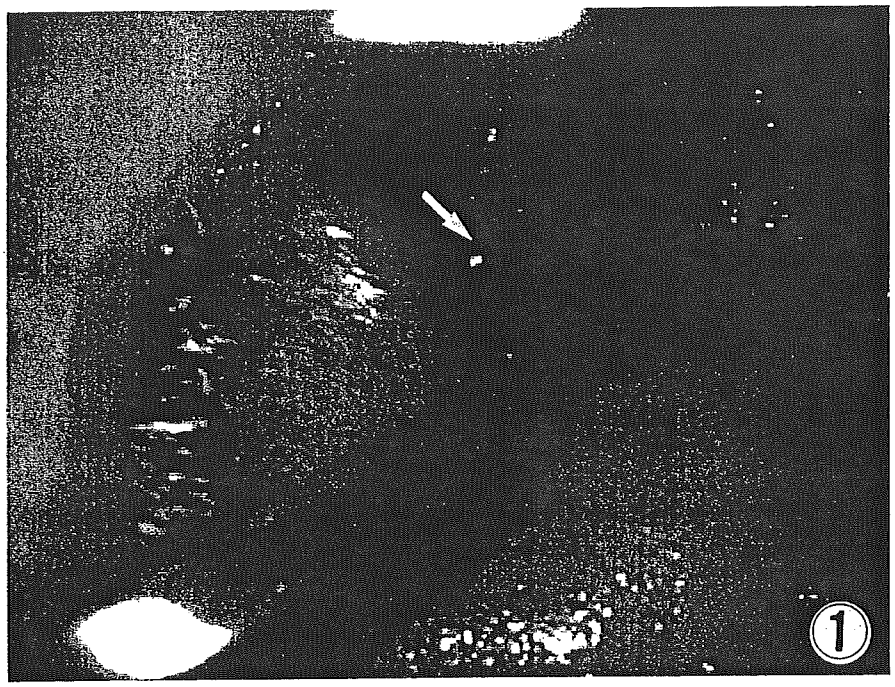
170



171

（図の説明）

図１．虫体摘出１ヶ月後の咽頭，矢印で虫体の寄生部位を示した．

図２．ホルマリン固定，未染色標本．

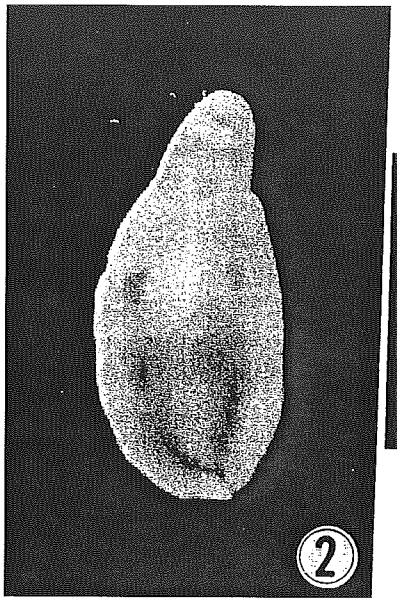
図３．体前部両側表面における微小な皮棘．

図４．カルミン染色標本．

図５．　　図４の模写図．

Os:口吸盤，A:腹吸盤，V:卵黄巣，Au:子宮の前部，Pu:子宮の後部，

O:卵巣，At:前精巣，Pt:後精巣，It:腸管，Ev:排泄嚢



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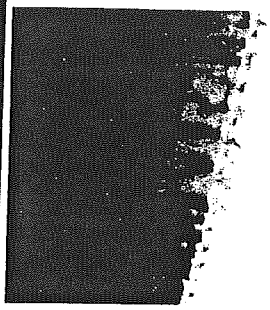

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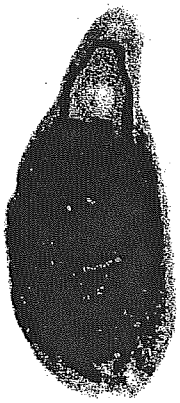

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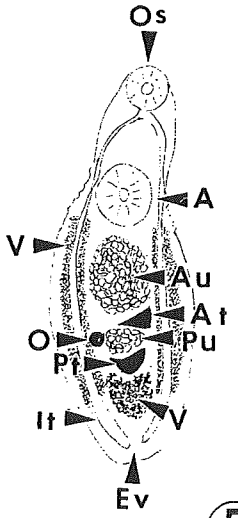

⑤

表　*Clinostomum* 人体咽喉頭寄生報告例(本邦)

	年齢	性別	住所	主訴	著者
1	22	F	大阪		Yamashita(1938)
2	38	F	富山	咽頭痛，咽喉頭異常感	堀(1942)
3	30	M	島根	咽喉頭異常感(疼痛感)	Kamo et al.(1962)
4	34	M	長崎	咽喉頭異常感，嚥下痛	坂口，他(1966)
5	53	M	岐阜	右側口腔違和感	佐野，他(1980)
6	31	F	愛知	発咳、血痰、疼痛	熊田，他(1983)
7	35	M	熊本	咽喉頭異常感(疼痛感)	Hirai et al.(1987)
8	57	F	滋賀	咽喉頭異常感，軽い嚥下痛	古川・宮里(1987)
9	29	F	福岡	咽頭部違和感	村田・高尾(1988)
10	15	F	島根	咽頭異物感	山根，他(1990)
11	54	F	佐賀	咽頭異物感，軽い嚥下痛	梅崎，他(1990)
12	70	F	秋田	咽頭痛	Yoshimura et al(1991)
13	68	M	島根	咽喉頭異常感	Isobe et al(1994)
14	37	F	佐賀	咽喉頭異常感	木船・上坂(1994)
15	40	F	佐賀	咽頭痛	木船，他(1994)
16	27	F	島根	咽頭異物感	前嶋，他(1996)
17	29	F	広島	咽頭違和感	白井，他(1998)
18	20	F	山口	咽喉頭異常感	木船，他(2000)
19	27	F	愛知	咽喉頭異常感	山本，他(2002)
20	55	F	群馬	咽頭部違和感	楾，他(2003)
21	27	M	佐賀	咽頭痛，咽喉頭異常感	柿添，他(2004)
22	46	F	熊本	咽頭痛，咽頭部違和感	自験例(2005)

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Altered expression of goblet cell- and mucin glycosylation-related genes in the intestinal epithelium during infection with the nematode *Nippostrongylus brasiliensis* in rat.

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Running head: Mucin response in nematode infection

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Summary

Intestinal nematode infection induces marked goblet cell hyperplasia and mucus secretion, but the mechanisms of regulation of the changes still remain to be elucidated. In the present study, epithelial cells were isolated from the rat small intestine at various times after *Nippostrongylus brasiliensis* infection, and the levels of expression of goblet cell- and mucin glycosylation-related genes were estimated by semi-quantitative reverse transcription (RT)-PCR. Among the genes investigated, mucin core peptide (MUC) 2, sialyltransferase (Siat) 4c and trefoil factor family (TFF) 3 were upregulated as early as 2-4 days post-infection, suggesting that they are associated with an early innate protective response. Seven days post-infection and thereafter, when the nematodes reached maturity, significant upregulation of MUC3, MUC4, resistin-like molecule β (Relmβ) and 3O-sulfotransferase (3ST)1 was observed, while 3ST2 expression levels increased after the majority of the worms were expelled from the intestine. Similar alterations of glycosylation-related gene expression were also observed in mast-cell-deficient *Ws/Ws* rats, suggesting that mast cells in the epithelium are not relevant to the upregulation of these genes. The present finding that the expression level of each goblet cell- or glycosylation-related gene was altered differently during the time course of infection indicates the progression of sequential qualitative changes in the mucus layer after infection.

Key words: mucin; glycosylation; goblet cell; intestine; *Nippostrongylus*

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Introduction

Intestinal nematode parasites such as ascaris and hookworms infect more than 1.3 billion people in the world. Although they cause relatively little mortality, infections result in high levels of morbidity that can result in developmental retardation in infected children (1). Although it has been clarified that Th2 cytokines and/or Th2 cytokine-dependent responses such as mastocytosis and eosinophilia might have crucial roles in protective immunity against certain nematode infections (2), further clarification of factors or mechanisms that are responsible for colonization of worms in and/or rejection of worms from the intestine is an issue of importance for developing more effective measures to combat intestinal parasites. Among factors of potential importance, particularly interesting factors are produced in and secreted from goblet cells, including mucins and other secretory peptides. In fact, intestinal nematode infection induces marked goblet cell hyperplasia and mucus secretion, which may be induced directly via the local release of bioactive factors or indirectly via the activation of host immune cells (3,4).

The nematode *Nippostrongylus brasiliensis*, a lumen-dwelling parasite, is a suitable model for studying human and other clinically relevant hookworms because of the similarities of habitat and life cycle (5). Interestingly, the mucus response which is induced by infection with *N. brasiliensis* has been suggested to be responsible for the rejection of nematodes from the intestine, whereas mast cells are not essential for expulsion of this parasite (3,4). It has been shown that alterations in the terminal sugars of goblet cell mucins are associated with *N. brasiliensis* worm expulsion from the intestine by studies of lectin-binding as well as by biochemical analyses (6-10). Recently, Knight *et al.* (11) and Pemberton *et al.* (12) reported that the expression levels of a variety of goblet cell- and/or mucin-related genes were altered during infection with *Trichinella spiralis* in mice. The results indicated that molecular changes that are

induced in the epithelial and/or mucus layer during nematode infection are a complicated series of changes whose mechanisms of regulation are virtually unknown, as are the roles of each molecule in mucosal protection.

We therefore attempted to determine by semi-quantitative reverse transcription (RT)-PCR whether there were alterations of the expression of some goblet cell-related genes in the small intestine during the time-course of *N. brasiliensis* infection in rats. The genes examined included those of mucin core peptides MUC2, MUC3 and MUC4 (13-16) and goblet cell-specific secretory peptides such as Relmβ (17) and intestinal trefoil factor (TFF3)(18,19). Because of the possible importance of the terminal structure of oligosaccharide chains of mucins and/or membrane glycoproteins for colonization and/or rejection of worms, we also examined the gene expression of sialyltransferase, sulfotransferase, fucosyltransferase and some histo-blood group transferases, which might modulate the terminal sugar chains of mucin and/or membrane glycoproteins (20-26).

MATERIALS AND METHODS

Animals, nematode infection and autopsy

Specific-pathogen-free male Brown Norway/Sea (BN) rats and male Fischer (F)-344 rats were purchased from SLC Inc. (Shizuoka, Japan). SPF male and female mast cell-deficient *Ws/Ws* rats were produced in our laboratory as described previously (27). Animals at 8 weeks of age were injected subcutaneously with 2,000 *N. brasiliensis* infective-stage (L3) larvae as described elsewhere (27). The animals were allowed to feed *ad libitum* throughout the experiment.

Preparation of intestinal epithelial cells

The animals were sacrificed with an overdose of ether after overnight fasting with free access to water. The separation of intestinal epithelial cells was carried out at 4°C in EDTA-Hanks' solution (Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution supplemented with 10 mM HEPES, pH 7.3, 1 mM DTT and 1.0 mM EDTA) as described elsewhere (28) with slight modifications. In brief, a piece of jejunum 18-22 cm from the pyloric ring and a piece of ileum 10-14 cm from the ileocecal junction, or a 4-cm-long segment of the proximal colon, were removed, opened longitudinally and cut into segments 1 cm in length. After a brief wash in PBS, 4 pieces of tissue were put into a 15-ml tube containing 4 ml of EDTA-Hanks' solution, and debris attached to the mucosal surface was removed by vigorously shaking the tubes 15 times by hand. The tissues were then transferred into another tube containing EDTA-Hanks' solution. After 75 min on ice with occasional agitation of the tissues by inverting the tubes, the epithelial cells were separated by 60 strokes of vigorous shaking of the tube by hand. After discarding the tissue, detached epithelial cells were collected by centrifugation at 600 x*g* for 3 min at 4°C, washed once with EDTA-Hanks' solution, and the cell pellets were stored at -80°C until use. Giemsa staining of the separated epithelial fractions showed not only epithelial cells, but also a small number of mononuclear cells, the majority of which we considered might have been intraepithelial lymphocytes. Histological examination of the tissue after collection of the epithelia showed that villus epithelium was separated completely, whereas the epithelial lining cells in the lower part of crypts were still attached to the tissue in approximately half of the crypts. In uninfected rats, the basal lamina of the epithelium was intact and lamina propria cells were retained in the tissue, but in animals after 10 days of infection, the basal lamina was partly obscured, indicating that some propria mucosal cells contaminated the epithelial fractions. Concerning the stomach, a mucosal scrape specimen of the glandular stomach was

prepared by scraping the mucosa with the edge of a glass slide and stored at -80°C until use.

Extraction of total RNA, cDNA synthesis, RT-PCR and relative quantification

Total RNA was extracted using TRIZOL Reagent (Life Technologies, Rockville, Maryland). Two-microgram aliquots of RNA were reverse transcribed in 20 μl of reverse transcription buffer containing 5 mM $MgCl_2$, 1 mM dNTP mixture, 1U/μl RNase inhibitor, 0.25 U/μl AMV reverse transcriptase and 0.125 μM oligo dT-adaptor primer (Takara RNA LA PCR kit, Takara Biomedicals, Osaka, Japan) at 42°C for 50 min. One-microliter aliquots of the synthesized cDNA were mixed with Sybr Green PCR master mix (Applied Biosystems, Foster City, CA) with appropriate primers and amplified using a real-time PCR system 7300 (Applied Biosystems, Foster, CA, U.S.A.). The sense and antisense primers used were:
5'-CGGATCCAATGGAACAGTGG-3' and 5'-TGCCACTGGTAGGATGATTG-3' for MUC2; 5'-GTTTCAACTCGACTGCCACC-3' and 5'-ATAGCTGCAGTTCTTGGAGG-3' for MUC3; 5'-GCGGAAGAGGAGTGGAGAAG-3' and 5'-AGATGGCCAGTAGCAAGAGG-3' for MUC4; 5'-TTCCTTCTCTCGCTGATGGT-3' and 5'-GCAGTGGCAAGTAGTTCCAT-3' for Relmβ; 5'-ATGGAGACCAGAGCCTTCTG-3' and 5'-TGGGATGCTGGAGTCAAAACA-3' for Tff3; 5'-CTACACCTCTGCGACTTGGT-3' and 5'-GGTTCTTGACAGCTCCCATC-3' for Siat4c; 5'-CCCTTTCCCTGAGATCCAGA-3' and 5'-CCGGCCTTTGGACTCATGTA-3' for 3ST1; 5'-CCCAGATCCACTTCGTCAGT-3' and 5'-AAAATTCCCGGAGCTGGTCT-3' for 3ST2; 5'-AGCAATGGCATGAGATGGTG-3' and 5'-TCTGGAAGGGTGAAGTTAGC-3' for FUT1; 5'-GGTGCCGGGAGAACATTAAT-3' and 5'-GAGAATCCGGAAGGGTGTAG-3' for FUT2; 5'-GATTTCCCTAGTGCTGCCTC-3' and 5'-GTTGTGGATACTCTTGGGCT-3' for FUT4; 5'-ATGTACAAGTGGCCAGCCTA-3' and 5'-GAATCTTCCCTTCCCCAGAG-3' for Lew 1. Abbreviated terms for each gene are listed in Table 1. The specificity of each amplified product was confirmed by dissociation analyses giving a single sharp

dissociation peak, the absence of the amplified product without reverse transcription, and the appearance of a band of the expected size on electrophoresis of the amplified product. For the amplification of β-actin, Actb primers (Rn00667869, Applied Biosystems) and TaqMan PCR master mix (Applied Biosystems) were used. For relative quantification, standard curves of the threshold cycle (Ct) of amplification of each target against log ng total RNA were created using cDNA samples which showed the lowest Ct value in preliminary runs, and relative quantification was performed for each sample. All quantified values were normalized to those of β-actin (quantified value for a certain target/quantified value for β-actin).

Tissue preparation for histology and goblet cell count

A segment of the jejunum 22-26 cm distal to the pyloric ring and a segment of ileum 6 -10 cm from the ileocecal junction were removed, opened longitudinally, fixed in 4% buffered formalin overnight and embedded in paraffin in such a position that histological sections could be cut perpendicular to the luminar surface. Five-micrometer sections were cut and the periodic acid-Schiff (PAS) reaction with hematoxylin nuclear staining was carried out. Ten villi, which were cut as nearly perpendicularly as possible, were selected per animal, and the numbers of goblet cells and numbers of epithelial nuclei in each villus were counted under a microscope. Goblet cell number/100 epithelial cells was calculated as [Number of goblet cells/number of epithelial nuclei] x 100. The average number of goblet cells/100 epithelial cells in 10 villi was used as the representative value in a given animal, and means and SE of 4 animals were calculated.

Worm counts

After removing the jejunal and ileal segments for separation of the epithelium and tissue section preparation, the numbers of worms in other parts of the small intestine were determined by the saline incubation method.

Statistical analysis

Student's *t*-test (2-tailed) was employed for statistical analysis; a P value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Expression of goblet cell- and glycosylation-related genes along the gastrointestinal tract

The relative expression levels of goblet cell- and mucin glycosylation-related genes in the normal BN rat gastrointestinal tract were examined by semi-quantitative RT-PCR (Table 1). Except for Lewl and 3ST2, the goblet cell- and mucin synthesis-related genes examined were expressed at significantly higher levels in the colon than in the jejunum. This may partly reflect the greater abundance of goblet cells in the colon than in the small intestine, in which absorptive cells are the major epithelial constituent, and is consistent with previous reports that MUC2 and MUC4 gene/protein expression levels were higher in the colon than in the small intestine (14,16). In the stomach, MUC2, MUC3 and MUC4 expression was undetectable, while abundant expression of glycosylation-related genes was observed. This is consistent with the fact that MUC5AC and MUC6 are the major mucin core peptides in the stomach (29).

Goblet cell hyperplasia during the course of Nippostrongylus brasiliensis *infection in the small intestine*

N. brasiliensis larvae reach the small intestine as early as 2-3 days after cutaneous infection, develop to sexual maturity, and begin to lay eggs by 7 days post-infection (PI). However, infection does not continue for a long time: in the normal rat, the majority of adult worms are rejected from the small intestine around 14 days PI by a T-cell dependent mechanism, leaving only a small number of residual worms in the intestine (5, 30). To examine the kinetics of goblet cell response during the time course of infection, BN rats were infected with 2,000 L3 larvae of *N. brasiliensis* and autopsied 7, 14 and 21 days PI. The numbers of worms recovered from the intestine excluding the intestinal segments used for tissue preparation and epithelial separation were 345.8 $\pm$ 180.4, 40.0 $\pm$ 17.1 and 42.2 $\pm$ 32.9 (average $\pm$ SD) after 7, 14 and 21 days of infection, respectively, showing that the majority of worms were rejected from the intestine by 14 days PI, but a small number of worms escaped the rejection and continued to parasitize the rat at least until 21 days PI. PAS staining of the jejunal and ileal tissue sections revealed goblet-cell hyperplasia 7 and 14 days PI, while goblet cell numbers decreased to preinfection levels by 21 days PI (Table 2).

Alterations of goblet cell- and glycosylation-related gene expression during the course of Nippostrongylus brasiliensis *infection in the small intestine*

At least 3 types of mucins are expressed in the small intestine: MUC2, which is restricted to goblet cells, MUC3, which is expressed in both columnar and goblet cells, and MUC4, which is expressed in columnar cells (13-16). RT-PCR analyses of gene expression in isolated epithelial cells of BN rats showed upregulation of MUC2, MUC3 and MUC4 in the jejunum, but not in the ileum after infection (Fig. 1), indicating that upregulation of mucin core peptide genes occurred mainly in the local mucosa, which

was parasitized by large numbers of worms. In F-344 rats, similar kinetics of gene expression were observed after nematode infection (data not shown). It has been reported that MUC2 and MUC3 mRNA expression was increased in the small intestinal epithelium after infection with the epithelium-invading nematode *T. spiralis* in mice (31). The present findings that not only MUC2 and MUC3, but also MUC4 mRNA expression was upregulated after infection suggest that the mucin response to nematode infection might have occurred not only in goblet cells, but also in columnar cells.

The gene expression of goblet cell-specific non-mucin peptide Relmβ, whose expression is dependent on Th2 cytokines and has a potentially important role in protective immunity (11,17), was also upregulated after infection (Fig. 1), consistent with previous reports showing that Relmβ expression was induced after infection of mice with *Trichuris muris, T. spiralis and N. brasiliensis* (11,17). The finding that strong upregulation of Relmβ occurred not only in the jejunum, but also in the ileum, where scarcely any parasites were found, may reflect the systemic effect of Th2 cytokines. TFF3, which might protect mucous epithelia from a range of insults and is known to contribute to mucosal repair (18,19), was also upregulated, consistent with its upregulation in *T. spiralis*-infected mice (32). In a previous report, TFF3 gene expression was not altered in mice infected with *N. brasiliensis* (33); the discrepancy may possibly be due to the difference of host species.

Not only the production and secretion of mucins and goblet cell-specific peptides, but also the glycosylation status of the terminal sugar chains of mucins and/or membrane glycoproteins might have an important role in the colonization of nematodes in and/or rejection of nematodes from the intestine. Although a variety of glycosyltransferases may be involved in the modulation of the terminal sugar chains of mucins and membrane glycoproteins, little is known about the dynamic changes of these genes after infection, except that the upregulation of Siat 4c, FUT2 or A-type

transferase has been reported in mice or rats after infection with *T. spiralis* or *N. brasiliensis* (9-11). The present results showed that the gene expression of Siat 4c, 3ST1, 3ST2, FUT2 and Lew1 was upregulated after infection (Fig. 1), showing for the first time that not only sialyltransferase and fucosyltransferase but also sulfotransferase gene expression changed during nematode infection. The slight upregulation of the FUT2 and Lew1 genes after infection suggests that some changes also occurred in the histo-blood antigens after infection. Although the upregulation of these genes was transient and the levels of the mRNAs returned to steady state levels by 21 days PI, 3ST2 expression was instead upregulated 21 days PI, suggesting that the expression of the two sulfotransferases is regulated by different mechanisms.

Several types of glycosylation-related genes, especially O-sulfotransferase genes, are considered to be specifically expressed in mast cells, which synthesize sulfated glycosaminoglycans such as heparin and/or chondroitin sulfate (34). It is well established that the number of mast cells increases not only in the propria mucosa, but also in the epithelium, after *N. brasiliensis* infection (35). Thus, intraepithelial mast cells contaminating the epithelial fraction might be responsible for the upregulation of 3ST1 and/or 3ST2 after infection. To clarify this point, we examined the gene expression in the epithelial fraction of *Ws/Ws* rats, which have a small deletion of the c-kit gene and consequently lack mast cells (35). As shown in Fig. 2, the expression of Relmβ, Siat4c and 3ST1 was upregulated 7 days PI, and that of 3ST2 was upregulated 21 days PI, indicating that mast cells are not relevant to the expression of these genes. These results indicate that the glycosylation status of mucins and/or membrane glycoproteins was modified markedly during the course of infection. Although the precise roles of these changes in inflammatory conditions still remain to be elucidated, some reports have indicated a crucial role of the sialylation and sulfation levels of mucins in *Strongyloides venezuelensis*-infected mice and rats, with the sulfation levels

in goblet cells affecting the establishment and distribution of the nematodes in the intestine (36, 37).

Since *N. brasiliensis* larvae reach the small intestine as early as 2-3 days PI, early responses of goblet cell- and mucin glycosylation-related genes after infection were examined. As shown in Fig. 3, MUC2, TFF3 and Siat 4c expression in the jejunal epithelium was upregulated as early as 2-4 days after infection.

Taken together, the present findings show that the expression of each goblet cell- and glycosylation-related gene was altered differently during the course of infection (Fig. 4). The early onset of Siat4c, MUC2 and TFF3 gene upregulation might be associated with innate immunity to pathogens or may be related to an acute phase reaction. MUC3, MUC4, Relmβ and 3ST1 gene upregulation, which occurred in the adult-worm colonization and rejection stages, might be regulated by different mechanisms from those of early activated genes, possibly through T-cell- or Th2 cytokine-dependent acquired immunity. Moreover, the upregulation of sialyl- and sulfo-transferase genes suggests that the sialylation and sulfation status of the mucins, membrane glycoproteins and/or histo-blood group antigens might be altered dramatically during nematode infections. The results imply that goblet cell and/or mucin responses to infection are a far more complicated series of changes than previously thought, involving the sequential expression of many factors that can individually or coordinately affect the colonization or rejection of pathogens. Further elucidation of the roles and mechanisms regulating the expression of each mucin and non-mucin secretory peptide and regulating the glycosylation status would lead to development of new strategies for the treatment or prevention of not only intestinal nematode infection but also infection with other pathogens.

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