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Lectin Binding Patterns and Immunohistochemical Antigen Detection in the Genitalia of *Tritrichomonas foetus*-infected Heifers

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Summary

Heifers inoculated intra-vaginally with *Tritrichomonas foetus* were examined after long-term infection (70 days) and short-term infection (20 days) by lectin-histochemical, immunohistochemical and cultural techniques. The organism was recovered from the genital tract and *T. foetus* antigens were detected immunohistochemically in the lumina of uterine glands and cytoplasm of vaginal subepithelial macrophages. An increase of galactosylated residues (galactose and N-acetyl galactose), binding to PNA, was observed in the genital epithelium (vagina, uterus and oviduct) from infected animals. In the oviductal epithelium of short- but not long-term infected heifers, mannose (binding to Con A) was detected, suggesting that the persistent presence of *T. foetus* and its virulence factors or inflammatory processes result in a change in the glycoproteins of the epithelial surface. The findings have implications for the adhesion of *T. foetus* to cells and for the pathogenesis of bovine trichomonosis.

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Keywords: cattle; lectin; oviduct; parasitic infection; *Tritrichomonas foetus*; uterus; vagina; *Biochemical abbreviations used*: Fuc, fucose; Gal, galactose; GalNAc, N-acetyl-galactosamine; Glc, glucose; GlcNAc, N-acetyl-glucosamine; Man, mannose; NeuNAc, N-acetyl-neuraminic acid

Introduction

Tritrichomonas foetus (*T. foetus*) is a sexually transmitted flagellated protozoon confined to the reproductive tract of bulls and cows that causes reproductive loss (Skirrow and BonDurant, 1988). Although *T. foetus* has been the subject of extensive investigations, the pathogenesis of infection in the female and how the organism causes reproductive failure are not fully understood. In females, *T. foetus* produces genital tract infection with inflammation and fetal loss. A minimal immune-inflammatory response occurs during early infection (Parsonson *et al.*, 1976; Skirrow and BonDurant, 1988). These authors reported vaginal antibodies to *T. foetus* at 42–56 days post-infection but no histological changes occurred in vaginal tissue before 60 days. Nevertheless, severe endometritis and fetal loss may occur at 63–70 days post-infection (Parsonson *et al.*, 1976; Anderson *et al.*, 1996). Several parasite-associated molecules, including neuraminidases, proteases, adhesins and cytotoxins, have been described in *T. foetus* (Skirrow and BonDurant, 1988; Kania *et al.*, 2001; Shingh *et al.*, 2001), but their role in pathogenesis and the mechanisms by which inflammation is suppressed during early infection require further investigation.

The technique of lectin binding relies on the affinity of these substances for specific terminal sugars and for α or β anomeric linkages to the penultimate residues (Spicer and Schulte, 1992). The method facilitates identification of complex

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structures to which the terminal sugar attaches and may reveal physiological or pathological changes in cells, intercellular interactions or intracellular transport pathways (Sharon and Lis, 1989; Spicer and Schulte, 1992). It has been suggested that modifications in glycoconjugates in the endometrium (Munson *et al.*, 1989) and oviduct (Abe *et al.*, 1995) of cattle play a role in failure of fertility or implantation, or both. Lectins have also been used to determine surface glycoconjugate changes in the tissues of heifers infected with *Campylobacter fetus* (*C. fetus*) subspecies venerealis (Cipolla *et al.*, 1998). The glycoconjugate response in *T. foetus*-infected genital tissue and its relationship to the inflammatory response and presence of the parasite is still unknown; it may, however, be relevant to pathogenesis.

The purpose of this study was to investigate the lectin-binding pattern, histological changes and immunohistochemical demonstration of parasites in the genitalia of *T. foetus*-infected heifers after both long- and short-term infection.

Materials and Methods

Animals and Experimental Procedure

Twelve postpubertal virgin heifers were divided into three groups (A, B and C) of four. Before the study commenced, the animals were "synchronized" with estrumate cloprostenol (Schering-Plough, Germany). To produce oestrus at the time of inoculation and thereby standardize the effects of the oestrous cycle. Heifers in groups A and B were inoculated intravaginally with 5×10^6 *T. foetus* (Cobo *et al.*, 2001). At 70 days post-inoculation, the animals of group A were killed for examination (long-term infection), and group B animals were killed at 20 days post-inoculation (short-term infection). Group C animals, which served as uninfected controls, were killed 23 days after synchronization.

Culture for T. foetus

Cervico-vaginal mucous secretions were collected at weekly intervals, cultured in liver infusion medium and examined by microscopy for identification of *T. fetus*. At necropsy, genital secretion samples obtained from the vagina, uterus and oviduct were cultured in a similar fashion (Cobo *et al.*, 2001).

Tissue Samples and Histopathology

Samples of vagina, glandular inter-caruncular endometrium and ampullar oviduct were collected for histological and histochemical analyses. The tissues were fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 5 μm, and stained with haematoxylin and eosin.

Immunohistochemistry

A commercial avidin-biotin complex kit (Vectastain ABC Elite, Rabbit IgG; Vector Laboratories, Burlingame, CA, USA) as used. Paraffin wax-embedded tissue sections (5 μm) were mounted on slides coated with 3-aminopropyltriethoxy-silane (P8920; Sigma Diagnostics, St Louis, MO, USA) and dewaxed with xylene. To quench endogenous peroxidase, the sections were incubated in H_2O_2 3% in methanol for 10 min at room temperature, and then passed through a graded alcohol series before rinsing three times in deionized water and phosphate-buffered saline (PBS; pH 7.2, 0.01 M). Protease enzyme (P1547; Sigma) 0.1% in Tris–buffered saline (pH 7.5; Dako, Carpinteria, CA, USA) was added for antigen retrieval. The slides were then given two 3-min rinses in PBS and treated with blocking solution (ABC kit) for 30 min at room temperature to block non-specific sites. Primary *T. foetus* antibody (1 in 500 dilution in PBS), prepared in rabbits according to Campero *et al.* (1989) and kindly supplied by R.H. BonDurant, University of California Davis, was applied for 45 min at 37°C. After three rinses in PBS, sections were incubated for 30 min at 37°C with secondary biotinylated rabbit anti-bovine IgG (Vector). After three additional rinses in PBS, peroxidase-labelled avidin was applied at 37°C for 30 min. Sections were then rinsed in PBS, and the enzyme activity was detected by treatment for 10 min with 3-amino-9-ethylcarbazole 3% in N,N-dimethylformamide (AEC Substrate-Chromogen System; Dako). After counterstaining with Mayer's haematoxylin, the slides were dehydrated and mounted (Crystal Mount; Biomeda Corporation, Foster City, CA, USA) for examination. *T. foetus*-infected fetal lung tissue was used as a positive control and non-immune normal rabbit serum was substituted for primary antibody as a negative control.

Lectin Histochemistry

Paraffin wax-embedded sections (5 μm) were used. After dewaxing, sections were treated with hydrogen peroxide 0.3% in methanol for 30 min at room temperature (to inhibit the endogenous peroxidase), rinsed several times in 0.01 M PBS (pH 7.2), and treated with bovine serum albumin 0.1% in PBS for 15 min. The sections were then incubated

for 1 h at room temperature with biotinylated lectins. Seven lectins (Lectin Kit BK 1000; Vector) with different specificity were used, as follows: Con A (Concanavalia ensiformis, binding specificity α-D-Man and α-D-Glc); DBA (Dolichos biflorus, binding specificity α-D-GalNAc); SBA (Glycine max, binding specificity α-D-GalNAc, α-D-GalNAc and α and β-Gal); PNA (Arachis hypogaea, binding specificity β-D-Gal and (1-3) Gal Nac); RCA-I (Ricinus communis-I, binding specificity β-D-Gal and α-D-Gal); UEA-1 (Ulex europaeus-I, binding specificity α-L-Fuc); and WGA (*Triticum vulgaris*, binding specificity α-D-GlcNAc and NeuNAc). The optimal lectin concentration was 30 μg/ml in PBS for all lectins, except PNA, which was applied at a concentration of 10 μg/ml. The slides were incubated with an avidin-biotin-peroxidase complex (ABC) (Vector) for 45 min. The horseradish peroxidase was activated by incubation for 1–2 min with a diaminobenzidine commercial kit (Dako). Specimens were rinsed in distilled water, dehydrated with graded ethanol solutions, cleared in xylene and mounted in Permount (Fisher Scientific International, Hampton, NH, USA). Controls for lectin staining included: exposure to horseradish-peroxidase and substrate medium without lectin; and blocking by incubation with the appropriate blocking sugars (0.1 to 0.2 M in PBS) for 1 h at room temperature before applying to the sections. The intensity of lectin binding was subjectively scored from 0 (none) to 3 (strongly positive).

Results

Cultural Examination for T. foetus

Experimentally infected heifers were culture-positive from the time of inoculation until they were killed, except for one heifer (group A, no. 1), which was positive until one week before death. At necropsy, *T. foetus* was found to have colonized the genital tract of seven of the eight inoculated heifers.

In group A, heifer 1 failed to yield the organism *post mortem*; in animals 2 and 3 *T. foetus* was isolated from the vagina, uterus and oviduct, and in animal 4 from the vagina and uterus but not oviduct. All three sites were culture-positive in the four group B heifers (short-term infection).

Gross Lesions and Histopathology

Animal 4 (group A) had pyometra and both uterine horns contained 150 ml of pale yellow, semi-solid material. No macroscopical change was noted in the remaining infected and non-infected heifers.

Microscopically, the vaginal mucosa of heifers with long-term infection (group A) displayed prominent multifocal lymphoid subepithelial aggregates. The vaginal mucosa showed hyperplasia of epithelial cells, a superficial diffuse infiltrate of mononuclear and plasma cells, and sometimes intraepithelial neutrophils and eosinophils. Mild epithelial dysplasia and degeneration were also noted.

The interplacentomal endometrium had an inflammatory infiltration of macrophages and plasma cells in the strata compactum and spongiosum. Multifocal nodular subepithelial lymphoplasmacytic infiltrates were typically present in the stratum spongiosum, especially adjacent to the endometrial glands. The endometrial glands showed a flatted epithelium. Near the myometrium they were dilated and clustered. Fibroblasts and mononuclear cells were found in periglandular locations, while neutrophils were located in the lumina.

Mild salpingitis with focal lymphoplasmacytic infiltration of the submucosa was common in group A animals. The genital lesions described were more severe in the heifer with pyometra (no. 4) than in animals 2 and 3. Heifer 1, which was culture-negative at necropsy, had only mild lesions of the genital tract.

In the sections of vaginal mucosa of heifers with short-term infection (group B) there was a moderate subepithelial infiltrate of lymphocytes and plasma cells with foci of lymphohistiocytic aggregates. Occasionally, mid-epithelial hyperplasia with neutrophilic surface exudation and exocytosis was present. No lesions were found in sections of uterus and oviduct of group B heifers or in the genital tracts of non-infected heifers (group C).

Immunohistochemistry

Labelled *T. foetus* organisms were seen in the lumina of uterine glands in heifers with long-term infection (group A), especially in animal 4 (Fig. 1A), but not in group B (short-term infection). Labelling of parasites was strong (scored 2–3) and diffusely distributed over each trichomonad, including the flagella and the undulating membrane. The organisms either adhered to the apical endometrial cell surface or were free in the lumen, singly or in clusters. In two of the four group B heifers, labelling was seen in the cytoplasm of subepithelial macrophages and giant cells of the vaginal mucosa (Fig. 2B).

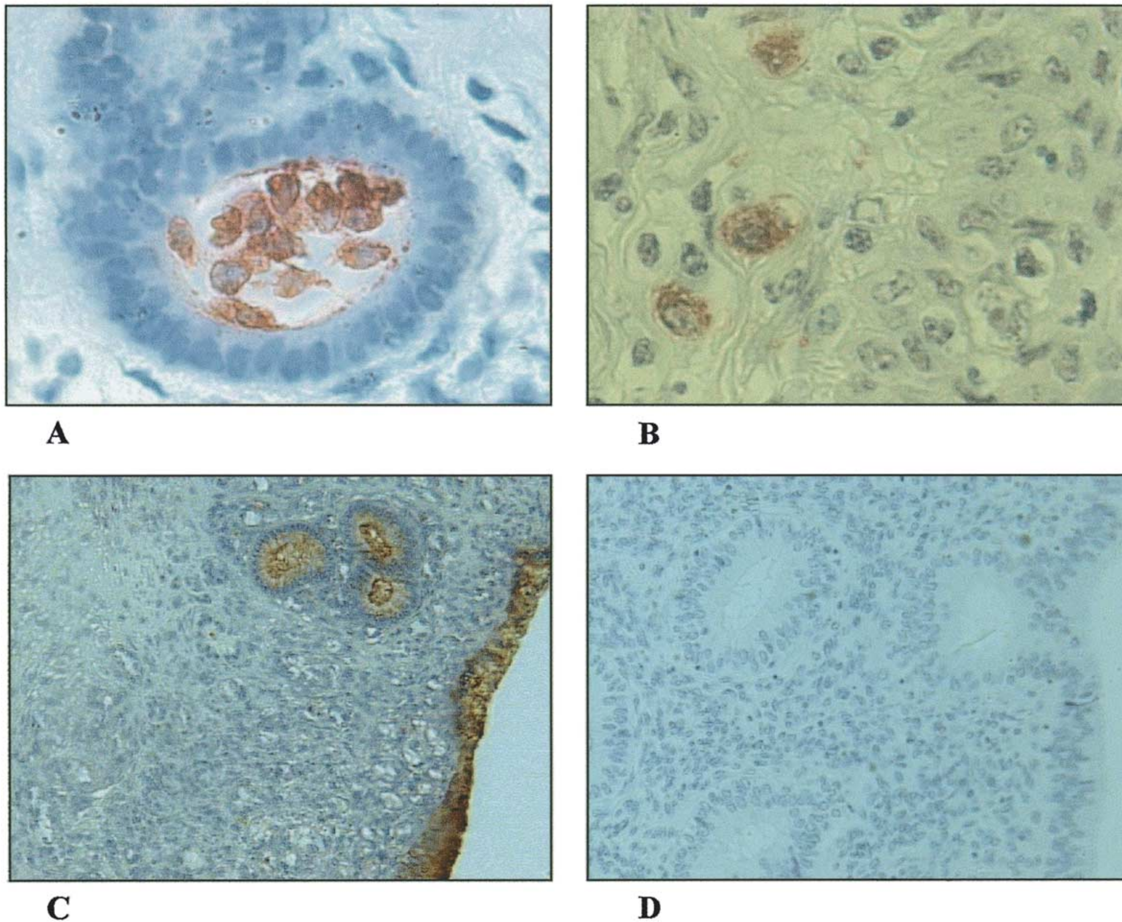

Fig. 1A–D. (A) Section of uterine gland of long-term infected heifer, showing *T. foetus* within the lumen. Immunohistochemistry. × 1000. (B) Section of vaginal mucosa of a short-term infected heifer. Cytoplasm of subepithelial macrophages shows reactivity with anti-*T. foetus* rabbit serum. Immunohistochemistry. × 1000. (C) Section of uterine epithelium of long-term infected heifer. UEA-1 binding to luminal and glandular epithelium. Lectin-histochemistry. × 400. (D) Section of uterine epithelium of non-infected heifer. Negative reactivity to UEA-1. Lectin-histochemistry. × 400.

Lectin Labelling

Lectin binding patterns for the seven lectins in various genital sites of the infected and non-infected animals are summarized in Table 1.

In the uterine glandular epithelial cells, UEA-1 bound with highest intensity in long-term infected heifers (group A), but weakly if at all in groups B and C. In the luminal epithelial cells, UEA-1 binding was clearly greater in groups A and B than in group C (Fig. 1C and D). In the infected heifers, intense PNA labelling was seen in the epithelial cells of the deep part of uterine glands (Fig. 2A); in the uninfected controls, however, such labelling was either absent or present only in the superficial parts of the glands (Fig. 2B).

In the vaginal epithelium, an increase in Con A and DBA labelling was observed in the infected groups (A and B) mainly in the apical surface of the luminal stratum, as compared with control tissues. However, Con A and DBA showed strong reactivity in all groups in the basal stratum of the vaginal epithelium. PNA bound strongly to the apical and basal strata in infected animals, but weakly if at all in non-infected animals (Fig. 2C and D). RCA -I showed intense labelling in the basal stratum in heifer 4 (group A), which had pyometra; in the other infected animals and the controls, however, the highest intensity of binding was seen in the apical stratum. SBA binding in the apical stratum was more intense in the infected heifers than in the controls.

In the epithelial cells of the oviducts, Con A binding was highest in group B heifers followed by group C, and lowest in group A. SBA and PNA gave intense labelling in infected heifers, but little if any in the controls (Fig. 2E and F). In the apical cytoplasm, RCA-I showed higher reactivity in group

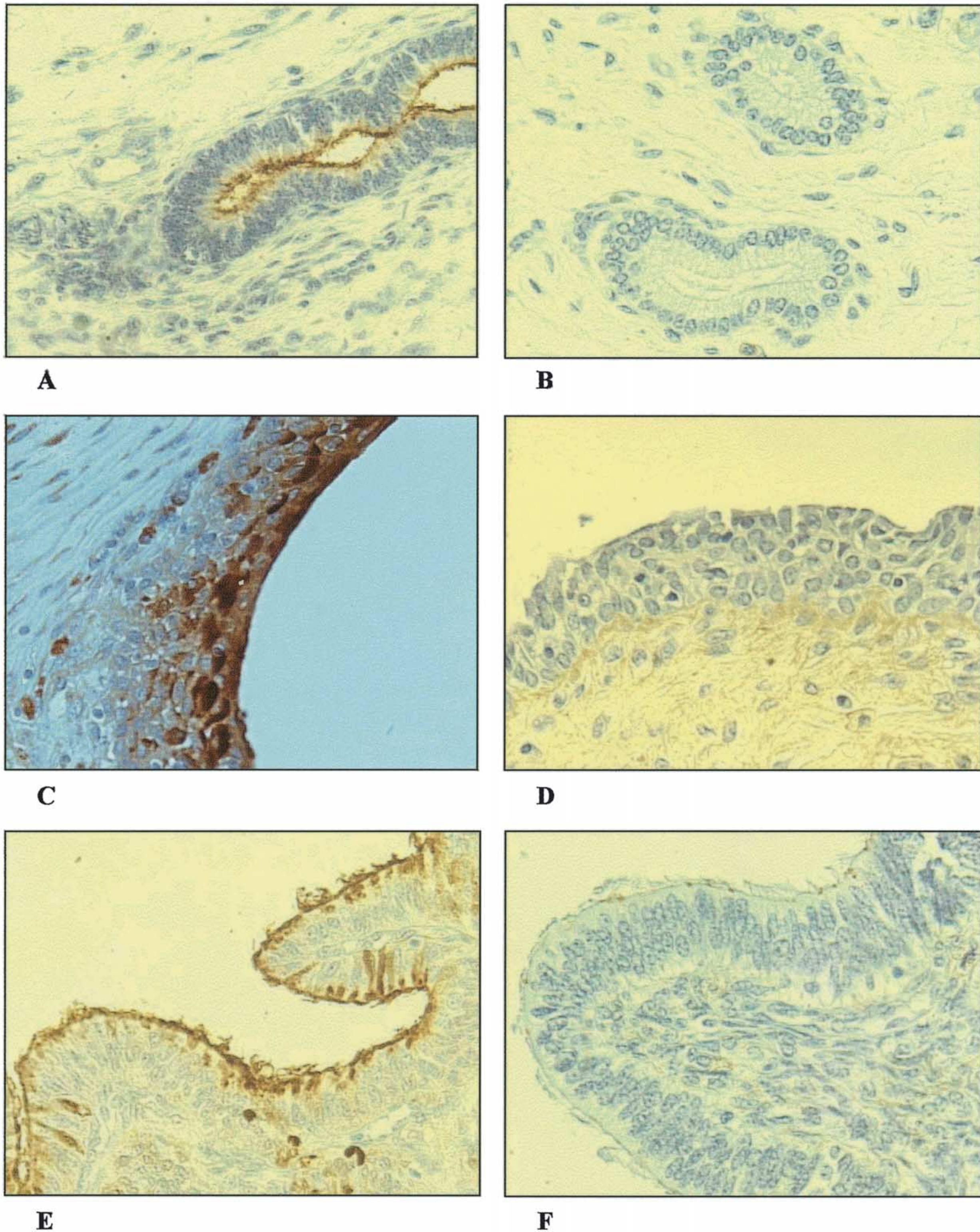

Fig. 2A–F. (A) Section of uterine mucosa of long-term infected heifer. PNA binding to glandular epithelium. Lectin-histochemistry. × 400. (B) Section of uterine mucosa of non-infected heifer. Negative reactivity to PNA. Lectin-histochemistry. × 400. (C) Section of vaginal epithelium of long-term infected heifer. PNA binding to epithelium. Lectin-histochemistry. × 400. (D) Section of vaginal epithelium of non-infected heifer. Negative reactivity to PNA. Lectin-histochemistry. × 400. (E) Section of oviductal epithelium of long-term infected heifer. PNA binding to epithelium. Lectin-histochemistry. × 400. (F) Section of oviductal epithelium of non-infected heifer. Negative reactivity to PNA. Lectin-histochemistry. × 400.

Table 1
Lectin binding in the genital tract of normal and *T. foetus*-infected heifers

Sites	*Intensity* of binding of the stated lectins in 3 groups (A, B, C) of heifers*																				
	Con A			*DBA*			*SBA*			*PNA*			*RCA-I*			*UEA-I*			*WGA*		
	A	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
Vagina																					
Apical stratum	3	3	1	3	3	1	3	3	2	3	3	1	3†	3	3	3	3	3	3	3	3
Basal stratum	3	3	3	3	3	3	2	2	2	2	2	0	2†	2	2	2	2	2	3	3	3
Uterus																					
Luminal epithelial cells																					
Apical cell surface	2	2	2	3	3	3	3	3	3	2	2	1	3	3	3	3	3	0–1	3	3	3
Supranuclear cytoplasm	2	2	2	3	3	3	2	2	2	2	2	1	1	1	1	3	3	0–1	2	2	2
Glandular epithelial cells																					
Apical cell surface	3	3	3	3	3	3	3	3	3	3‡	3‡	0–1§	3	3	3	3	0	0–1	3	3	3
Supranuclear cytoplasm	3	3	3	3	3	3	2	2	2	3‡	3‡	0–1§	1	1	1	3	0	0–1	1	1	1
Oviduct																					
Epithelial cells																					
Apical cell surface	1	3	2–3	1	1	2	3	3	0–1	3	3	1	3	3	3	1	1	0	3	3	2
Supranuclear cytoplasm	1	3	2–3	1	1	1	3	3	0–1	3	3	1	2	3	0–1	0–1	0–1	0	1	1	1

A, long-term infection group; B, short-term infection group; C, uninfected (control) group.
*Scored as 0 (nil) to 3 (strongly positive).
†Heifer 4 (in group A) scored 1 in apical stratum and 3 in basal stratum.
‡In the deeper parts of the uterine glands.
§In the superficial parts of the uterine glands.

B heifers than in group A or C animals. DBA labelling was highest in the apical cell surface of control animals.

Discussion

The present lectin histochemical study, supported by culture, immunohistochemistry and histopathology, revealed a distinctive pattern of oligosaccharide distribution in the genitalia of *T. foetus*-infected heifers.

At 70 days post-infection, endometritis was present, with aggregation of mononuclear cells, lymphoid foci in the submucosa, and periglandular fibrosis; moreover, *T. foetus* was detected immunohistochemically. Such changes have previously been associated with fetal loss. The presence of lymphoid aggregates adjacent to the deeper uterine glands and the absence of major uterine lesions in the short-term infected heifers accord with earlier studies (Parsonson *et al.*, 1976; Anderson *et al.*, 1996; Corbeil *et al.*, 1998).

The finding of immunohistochemically labelled material in the cytoplasm of vaginal subepithelial macrophages in short-term infected heifers is noteworthy, implying that *T. foetus* antigen is taken up by macrophages, leading to epitope presentation. Similar phagocytosis of *T. foetus* antigens by macrophages or neutrophils has been described in fetal lung (Rhyan *et al.*, 1995), and subcutaneous tissues of mice (Campero *et al.*, 1989). Failure to demonstrate immunolabelled *T. foetus* in other culture-positive organs appears to confirm the lability of the protozoon in superficial locations on the genital epithelium of heifers and bulls (Campero *et al.*, 1989). The identification of macrophages containing *T. foetus* antigens supports the hypothesis proposed by Corbeil *et al.* (1998), which may be stated as follows. Antigen in the first instance is taken up by vaginal epithelial cells and presented by macrophages or Langerhans cells below the epithelium. After a considerable period without severe lesions, stimulated B cells enter lymphoid follicles, proliferate and differentiate into plasma cells that reach the vaginal lamina propria (Corbeil *et al.*, 1998). In long-term infection antigen-presentation and the presence of *T. foetus* may have ceased; however, a focal lymphoid reaction in the subepithelium may remain, as previously described (Parsonson *et al.*, 1976; Anderson *et al.*, 1996; Corbeil *et al.*, 1998).

Regarding the observed lectin staining patterns, a common finding in the vagina, uterus and oviduct from infected and non-infected heifers was reactivity to WGA, a characteristic of cells that specialize in moving fluid and ions across membranes (Spicer and Schulte, 1992). The binding of PNA to the endometrial epithelium in all animals indicated an increase in Gal and GalNAc.

These sugars play a role in the adhesion of chorion to endometrium (Munson *et al.*, 1989).

O-linked oligosaccharides containing GalNAc indicate secretory and transport functions in the uterus (Munson *et al.*, 1989; Spicer and Schulte, 1992), and may be produced by the embryo or by *T. foetus* cells. Moreover, the fact that PNA binding was higher in the depth of the infected glands suggests that *T. foetus* migration into the glands increased the production of GalNAc glycoconjugates. Similarly, PNA increase in luminal and glandular epithelial cells was observed in the bovine venereal disease caused by *C. fetus* venerealis (Cipolla *et al.*, 1998).

Terminal L-Fuc was detected at very low levels in normal heifers but was invariably present at the luminal surface in infected animals. Additionally, it was also present in the glands of long-term but not short-term infected animals. This may be related to restriction of the protozoa to the surface epithelium in short-term infection and to their location in the glands in long-term infection.

In oviducts of group A animals galactosylated residues recognized by SBA, RCA-I and PNA were detected. This finding accords with a study in which oviductal epithelium of postmenopausal women showed intense reactivity with PNA but none with Con A (changes associated with failure of cell to cell adhesion) (Gheri *et al.*, 2001).

The enzyme neuraminidase has been isolated from *T. foetus*, *T. mobilensis* and *T. vaginalis*. It hydrolyzes the α-2,3-linked glycosidic linkage between sialic acid and underlying sugars from glycoconjugates (Engstler and Schauer, 1993; Padilla-Vaca and Anaya-Velázquez, 1997; Babál *et al.*, 1999). In the present study, high PNA activity (detecting galactosylated residues) in the vagina, uterus and oviduct of infected animals suggests that neuraminidase acts on the genital epithelium, revealing internal glycoconjugates (specifically, β-D-Gal of [1,3] GalNac, penultimate to sialic acid). This removal of sialic acid may favour *T. foetus* cell adhesion and consequent modification of the target cell surface, possibly a prerequisite for the degradation of glycoconjugates by other glycosidases and proteases (Bonilha *et al.*, 1995; Padilla-Vaca and Anaya-Velázquez, 1997; Babál *et al.*, 1999).

A further virulence factor that may be responsible for changes in lectin pattern is the extracellular cysteine proteinase, isolated from the surface of *T. foetus* and *T. vaginalis* (Arroyo and Alderete, 1989; Talbot *et al.*, 1991; Thomford *et al.*, 1996; Kania *et al.*, 2001). Cysteine proteinase cleaves lactoferrins of bovine cervical mucus (Talbot *et al.*, 1991) and the α chain of bovine complement C3 (Kania *et al.*, 2001). The high expression of Gal, GalNAc, and L-Fuc in the lumen of uterine glands at 70 days after infection may have been associated with cysteine proteinase, previously linked to inflammatory responses and high concentration of cleaved C3 (Kania *et al.*, 2001). The results of the present study, together with the descriptions of *T. foetus* virulence factors (Burgess and McDonald, 1992; Thomford *et al.*, 1996; Babál *et al.*, 1999; Kania *et al.*, 2001; Shing *et al.*, 2001) represent an advance in the understading of bovine trichomonosis.

In conclusion, an increase in galactosylated residues was demonstrated in the genital epithelia of *T. foetus*-infected heifers. The changes in lectin binding pattern may have been the consequence of either an inflammatory reaction or the effects of *T. foetus* enzymes such as neuraminidase and cysteine proteinase.

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