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EXPERIMENTALLY INDUCED DISEASE

Lectin Binding Pattern in the Uterus of Pregnant Mice Infected with *Tritrichomonas foetus*

M. A. Woudwyk[*], E. J. Gimeno[†], P. Soto[‡], C. G. Barbeito[*,†] and C. E. Monteavaro[‡]

[*] *Laboratory of Histology and Embryology, Faculty of Veterinary Sciences, National University of La Plata, La Plata,* [†] *Institute of Pathology, Faculty of Veterinary Sciences, National University of La Plata, La Plata and* [‡] *Laboratory of Clinical and Experimental Microbiology, Faculty of Veterinary Sciences, National University of the Centre of Buenos Aires Province, Tandil, Buenos Aires, Argentina*

Summary

Bovine genital tritrichomonosis is caused by the protozoon *Tritrichomonas foetus* and leads to embryonic death and abortion. The complexity of handling bovine experimental systems has led to the development of alternative models. The infection has been reproduced in pregnant BALB/c mice. In the pathogenesis of the disease, adhesion of the protozoon to host cell surface glycoproteins is important. Labelling with soya bean agglutinin (SBA) and peanut agglutinin (PNA) lectins increases in the luminal and glandular uterine epithelium of non-pregnant infected mice. The aim of the present study was to determine whether these changes also occur in pregnant infected BALB/c mice. Female BALB/c mice were inoculated intravaginally with *T. foetus* and, 15 ± 3 days post infection, were paired with males overnight. Infected and control mice were sacrificed 6, 8 and 10 days later. Samples of uterus were labelled with a panel of biotinylated lectins. Infected mice showed increased binding of PNA and SBA. There was also increased binding of concanavalin (Con-A) by luminal epithelium and *Ricinus communis* agglutinin (RCA-1) by glandular epithelium at day 6 post coitum. These changes may be due to the production of enzymes by *T. foetus*, which could act to enhance adhesion and colonization and thus favour infection.

Keywords: bovine genital tritrichomonosis; lectin histochemistry; mouse model; pathogenesis

Bovine genital tritrichomonosis (BGT) is a sexually transmitted disease caused by the flagellated protozoon *Tritrichomonas foetus*. This parasite colonizes the reproductive tract of cows, triggering temporary infertility, early embryonic death and, in some cases, abortion (BonDurant, 2005).

Laboratory animal models represent an excellent tool for unravelling the pathogenesis of the infection (St Claire *et al.*, 1994; Hook *et al.*, 1997). BALB/c mice are the most susceptible laboratory animal and the disease can be reproduced in this strain using low-dose oestrogen to synchronize oestrous (Soto *et al.*, 2005). Genital tritrichomonosis has also been induced in pregnant BALB/c mice treated with a low dose of oestrogen (Agnew *et al.*, 2008; Barbeito *et al.*, 2008).

Lectin histochemistry may be used to detect changes in the expression of carbohydrates on cell surfaces. Cobo *et al.* (2004) reported an increase of galactosylated residues, binding to peanut agglutinin (PNA), in the genital epithelium of heifers infected with *T. foetus*. Monteavaro *et al.* (2008) have demonstrated that in non-pregnant BALB/c mice infected with *T. foetus*, endometrial epithelium shows an increase in the intensity of labelling with PNA and soya bean agglutinin (SBA). The aim of the present study was to determine the nature of carbohydrates expressed by luminal and glandular epithelia of the endometrium of pregnant mice infected with *T. foetus* and to compare this expression with that shown previously in heifers and non-pregnant BALB/c mice.

Correspondence to: E. J. Gimeno (e-mail: ejgimeno@fcv.unlp.edu.ar).

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Female BALB/c mice, 6–8 weeks old, were housed according to the regulations of the National Administration of Medicine, Food and Medical Technology (ANMAT, 1996) with controlled temperature and airflow. Food and water were available *ad libitum*. Lighting was provided on a 12 h light/dark cycle. In order to synchronize oestrous, the animals were injected intramuscularly with 5 μg of β-oestradiol 3-benzoate suspended in 0.1 ml of sterile sesame oil. To confirm synchronization of oestrous, vaginal cytology was performed 24 and 48 h later. Two days after cycle synchronization the infected group ($n = 17$) was inoculated intravaginally with 10 μl of a suspension containing 9×10^7 *T. foetus*/ml (Soto *et al.*, 2005), while the control group ($n = 14$) was given phosphate buffered saline (PBS) by the same route. Twelve days later vaginal cytology was again analyzed in order to determine the phase of the oestrous cycle and to confirm the presence of *T. foetus* in the infected animals. The female mice in oestrous were paired with healthy BALB/c males. The presence of a vaginal plug was considered as day 0 of pregnancy. Five infected and five control mice were killed at 6 days post coitum; seven infected and four control mice were killed at 8 days post coitum and five infected and five control mice were killed at 10 days post coitum.

Uterine horns were removed from each mouse, fixed in 10% neutral buffered formalin and embedded in paraffin wax. Infected and control groups were subdivided into pregnant and not pregnant animals based on the presence of implantation sites (Table 1). Sections (5 μm) were stained with haematoxylin and eosin (HE) or subjected to lectin histochemistry (Monteavaro *et al.*, 2008). Seven biotinylated lectins (Lectin Kit BK 1000; Vector Laboratories, Burlingame, California, USA) with different carbohydrate specificity were used. These included concanavalin A (*Concanavalia ensiformis*; Con-A; binding specificity a-D-Man and α-D-Glc), *Dolichos biflorus* agglutinin (DBA; binding specificity α-D-GalNAc), SBA (*Glycine max*; SBA; binding specificity α-D-GalNAc and α and β-Gal), PNA (*Arachis hypogaea*; PNA; binding specificity β-D-Gal and β [1-3] GalNAc), *Ricinus communis* agglutinin-1 (RCA-1; binding specificity β-D-Gal and α-D-Gal), *Ulex europaeus* agglutinin-1 (UEA-1; binding specificity α-L-Fuc) and wheat germ agglutinin (*Triticum vulgaris*; WGA; binding specificity α-D-GlcNAc and NeuNAc). Controls for lectin labelling included exposure to horseradish peroxidase and substrate medium without lectin and blocking by preincubation with the appropriate blocking sugars (0.1–0.2 M in PBS) for 1 h at room temperature before applying lectin to the sections. Sections were examined with a light microscope (×40 objective) and the intensity of lectin binding was scored subjectively as 0, negative; 1, weakly positive; 2, moderately positive; and 3, strongly positive.

Table 1
Groups of animals

Days post coitum	*Control group*		*Infected group*	
	Pregnant	*Non-pregnant*	*Pregnant*	*Non-pregnant*
6	3	2	0*	5
8	3	1	4	3
10	4	1	1	4
Total	10	4	5	12

*No female sacrificed at 6 days post coitum was pregnant.

Pregnant females infected with *T. foetus* suffered a higher incidence of embryo lost or abortion, identified by the small size and the haemorrhagic or inflammatory appearance of the sites of resorption, when compared with control pregnant females. The histopathology of the uterus of infected animals showed parasites in the lumen of the endometrial glands. An infiltration of inflammatory cells including neutrophils, eosinophils, lymphocytes, macrophages and plasma cells was observed. None of the infected females killed at 6 days post coitum was pregnant. Three of seven pregnant mice killed at 8 days post coitum, which were infected, but then became negative for the parasite, had healthy embryos without signs of abortion. One pregnant mouse killed at 8 days post coitum was pregnant, but the placenta showed marked pathological changes. The remaining three animals in this group had lost their embryos. Microscopically, there was epithelial metaplasia and numerous apoptotic cells were noted in the luminal and glandular epithelium. The endometrial glands were dilated and contained a purulent exudate. At 10 days post coitum, one of five infected mice was pregnant, but the placenta showed haemorrhagic areas. The remaining four animals lost their embryos and had pyometra, oedema of the lamina propria of the endometrium, epithelial metaplasia and dilated glands containing *T. foetus* organisms and exudate.

The lectin binding patterns in the uterus of the infected and control mice are summarized in Table 2.The composition of carbohydrates in the endometrium of mice infected with *T. foetus* showed differences when compared with control mice. The lectin binding pattern was analyzed in the luminal epithelium of the endometrium as well as in the epithelium of the endometrial tubular glands. In each location, the lectin binding pattern of the cytoplasm and the apical glycocalyx was analyzed. PNA and SBA lectins, which both have specificity for N-acetylgalactosamine and β-D-galactose, showed the most obvious

Table 2
Lectin binding pattern in the uterus of normal and *T. foetus*-infected pregnant BALB/c mice

Lectin	*Labelled area*	*DAY 6*				*DAY 8*				*DAY 10*			
		Control group		*Infected group*		*Control group*		*Infected group*		*Control group*		*Infected group*	
		Pregnant	*Non-pregnant*	*Pregnant*	*Non-pregnant*	*Pregnant*	*Non-pregnant*	*Pregnant*	*Non-pregnant*	*Pregnant*	*Non-pregnant*	*Pregnant*	*Non-pregnant*
Con-A	GLE	2	2	–	3	2	3	2	3	3	2	3	3
	SCLE	1	1	–	2	1	1	2	2	2	1	1	1
	GCE	2	2	–	2	2	2	3	2	2	2	2	3
	SGE	1	1	–	1	1	2	1	2	3	1	1	1
RCA-I	GLE	2	2	–	3	2	3	2	2	3	3	2	3
	SCLE	0	0	–	2	1	1	1	0	1	1	1	0
	GCE	2	2	–	2	2	2	1	1	2	2	2	2
	SGE	1	1	–	1	1	1	0	0	1	0	1	1
WGA	GLE	3	2	–	3	2	3	3	3	2	2	2	3
	SCLE	1	1	–	2	0	3	1	1	2	1	0	1
	GCE	2	3	–	2	2	3	3	2	1	1	3	3
	SGE	1	1	–	1	1	3	1	1	1	1	2	2
PNA	GLE	0	1	–	2	0	0	1	1	0	1	3	1
	SCLE	0	0	–	1	0	0	0	2	0	1	0	0
	GCE	0	0	–	2	0	1	1	1	0	1	0	1
	SGE	0	0	–	2	0	0	0	1	0	0	0	0
SBA	GLE	0	1	–	1	0	0	0	1	1	2	0	1
	SCLE	0	0	–	0	0	0	0	0	1	0	0	0
	GCE	0	0	–	2	0	1	0	1	0	0	0	1
	SGE	0	0	–	1	0	1	0	1	0	0	0	0
UEA-I	GLE	0	1	–	1	0	0	0	2	0	3	1	2
	SCLE	0	1	–	1	0	0	0	2	0	3	0	1
	GCE	0	1	–	2	0	0	0	2	0	3	0	2
	SGE	0	1	–	2	0	0	0	2	0	3	0	1
DBA	GLE	0	0	–	1	0	1	0	0	0	1	1	0
	SCLE	0	0	–	1	0	1	0	0	0	1	1	0
	GCE	0	0	–	1	0	1	0	0	0	1	0	0
	SGE	0	0	–	1	0	1	0	0	0	1	0	0

–, not tested; 0, negative; 1, weakly positive; 2, moderately positive; 3, strongly positive; GLE, glycocalyx of the luminal epithelium; SCLE, supranuclear cytoplasm of the luminal epithelium; GGE, glycocalyx of the glandular epithelium; SCGE, supranuclear cytoplasm of the glandular epithelium.

changes in binding pattern. Labelling with PNA and SBA lectins was more intense in endometrial luminal and glandular epithelia of the infected mice at all stages of pregnancy when compared with controls (Fig. 1). For other lectins, the results varied according to the days post coitum and the glandular structure. The affinity for Con-A was augmented at 6 days post-coitum in the luminal epithelium of the uterus. In the case of WGA, RCA-1, UEA-1 and DBA, the results were more heterogeneous. DBA lectin is known to be a useful marker for uterine natural killer (NK) cells (Chen *et al*., 2012). DBA lectin allowed us to detect and clearly identify NK cells in the decidua basalis and myometrium of the uteri of the pregnant mice. These cells were also labelled with SBA lectin (Fig. 2).

In countries where natural breeding occurs, BGT remains an endemic disease. It has been reported in Argentina, Spain, Canada, Mexico, Costa Rica, Australia and in the USA (Campero and Cobo, 2006). Understanding the pathogenesis of the infection may help achieve its control in the future.

The histopathological findings described in the present study concur with those described previously in pregnant mice (Agnew *et al*., 2008; Barbeito *et al*., 2008) and are similar to those found in pregnant cattle (BonDurant, 2005). Most BALB/c mice infected with *T. foetus* and killed early in pregnancy show embryonic death without inflammatory changes in the uterus, suggesting a pathogenic mechanism that does not involve direct tissue damage (e.g. changes in substances involved in cellular adhesion between the trophoblast and the uterine epithelium) (Barbeito *et al*., 2008; Woudwyk *et al*., 2012).

T. foetus is known to produce proteases rich in cysteine. Such enzymes may alter the adhesion molecules of the trophoblast (Thomford *et al*., 1996). Other enzymes produced by the parasite, such as β-glucosidase, β-N-acetylglucosaminidase and α-mannosidase, exert

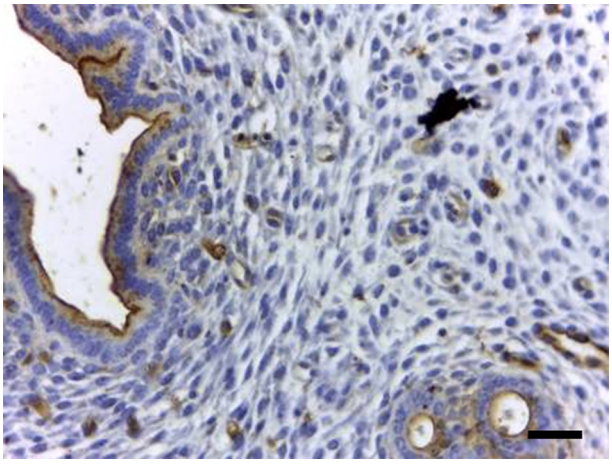

Fig. 1. Lectin histochemistry in the uterus of *T. foetus*-infected BALB/c mice at day 6 postcoitum. There is strong PNA binding in the glycocalyx of the luminal epithelium and moderate binding in the glandular epithelium. Bar, 20 μm.

their action on the mucus of genital organs such as the vagina, and create a hostile environment that would favour the pathogenesis of tritrichomonosis (Felleisen, 1999; Shingh *et al.*, 2001). These enzymes modify carbohydrates and so could be responsible for the changes observed in the lectin binding pattern in the female genital organs. Cell adhesion is a key process in the pathogenesis of the disease and the glycoproteins found on the surface of the protozoon and in the superficial epithelium of the host genital tract are essential in this interaction (Felleisen, 1999).

In heifers (Cobo *et al.*, 2004) and in non-pregnant BALB/c mice (Monteavaro *et al.*, 2008), infection with *T. foetus* changes the carbohydrate expression pattern in the luminal and glandular epithelia of the uterus. The most important changes are related to lectins that show affinity for N-acetylgalactosamine and β-D-galactose. In pregnant mice infected with *T. foetus* there is a greater exposure of galactosylated residues and consequently a greater affinity for SBA and PNA. These changes may facilitate the adhesion of the protozoa to the epithelia of the genital tract and secondarily generate alterations in implantation and placentation, leading to early embryonic loss.

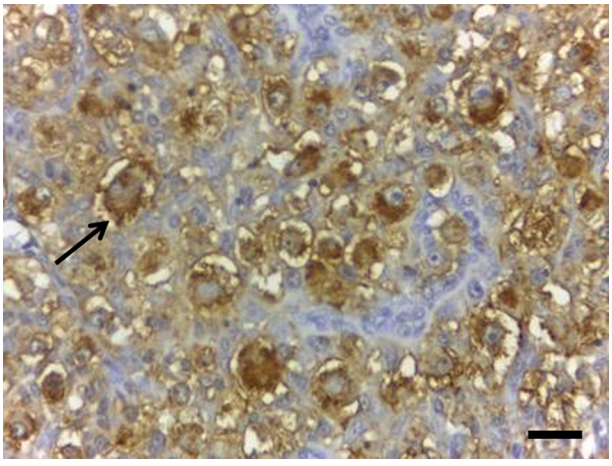

Fig. 2. Uterine natural killer cells (arrows) labelled with SBA lectin. Bar, 20 μm.

Uterine NK cells differentiate at implantation sites during murine pregnancy (Peel, 1989) and contribute to decidual and vascular remodelling at implantation sites. A useful marker for uterine NK cells is the DBA lectin, which binds specifically to terminal N-acetylgalactosamine (GalNAc) (Paffaro *et al.*, 2003). In the present study, DBA^+ uterine NK cells were identified in the decidua basalis and the myometrium. In pregnant mice, uterine NK cells were also labelled with SBA. There have been no previous reports of SBA labelling murine uterine NK cells. As for DBA, the SBA lectin binds specifically to terminal α-D-GalNAc. Further studies are needed to determine how these cells behave in tritrichomonosis.

The fact that in both pregnant and non-pregnant BALB/c mice the glycosylation changes are similar to those found in cattle supports the use of these animals as an experimental model for BGT research. These changes in glycosylation pattern may also be important in the pathogenesis of the disease.

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Conflict of Interest Statement

No competing financial interests exist for any of the co-authors of this manuscript.

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