The distribution of interstitial cells of Cajal in the ileum is not altered by infection with *Schistosoma mansoni*

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**Abstract**

*Background & objectives:* The interstitial cells of Cajal (ICC) act as pacemakers that generate slow waves and function as a relay between smooth muscle cells of the gastrointestinal (GI) tract. Recent reports indicate the crucial role played by the ICC in defining GI motility during human disease status like pyloric stenosis, Hirschsprung’s disease and ulcerative colitis. Experimental data showed that *Nippostrongylus* infection in the rat caused an altered GI motility pattern accompanied by a complete loss of ICC-deep muscular plexus. The aim of the present study was to delineate if ICC were similarly affected during *Schistosoma mansoni* infections, thereby responsible for the disturbed GI motility patterns triggered in the afflicted mammalian host.

*Methods & results:* Immunohistochemistry was done using whole mounts and sections from naïve and *S. mansoni* infected mice ileum. Primary antibodies detected Kit-immunoreactivity (Kit-ir representing ICC), PGP-9.5 (protein gene product 9.5 representing a neuronal marker), SK3 (ionic channel marker for non-Kit fibroblast like cells), and Cx43 (gap junction protein representing a muscle marker). Single/double immunofluorescence staining and confocal microscopy depicted that muscle thickness (Cx43-ir) and inflammatory infiltrate increased with infection. Kit-ir ICC and SK3-ir fibroblast like cells (FLC) were present at all normal locations as seen in controls and during acute and chronic stages of infection.

*Interpretation & conclusion:* No disappearance of either ICC population was noted. A preferential (although not exclusive) location of inflammatory infiltrate in contact with SK3-ir FLC in the muscle layer was observed. The present study thus delineated that ICC are not affected during *S. mansoni* infections, and thereby may not be responsible for mediating the disturbed GI motility patterns caused by schistosomiasis.

**Key words** Cajal interstitial cells – gut inflammation – *Schistosoma mansoni*

**Introduction**

Schistosomiasis is a parasitic disease caused by infection with trematodes belonging to the genus *Schistosoma*. *Schistosoma mansoni* infections are prevalent in Africa and South America. Adult *S. mansoni* worms live in the mesenteric veins around the human intestine as a couple, the male carrying the female in a gynaecophoric canal. The adult female worm produces between 300 and 3000 eggs per day. The eggs penetrate the tissues actively by means of proteolytic enzymes and eventually lodge themselves within...
the gut wall. Egg antigens secreted into the surrounding tissue trigger granulomatous, inflammatory responses consisting of macrophages, eosinophils and lymphocytes. This inflammation is responsible for the intestinal pathology associated with this disease.

In acute as well as chronic *S. mansoni*-induced gut inflammation, both structural and functional changes occur in the ileum. These changes are characterised by increased ileal wall thickness and smooth muscle contractility restricted to the inflamed gut segment. The overall hypercontractility disturbs the integrated propulsive activity of the normal gut, leading to a dysfunction of peristalsis and gastrointestinal (GI) transit.

*Schistosoma*-mediated inflammation of the GI tract involves the enteric nervous system. The effect of *S. mansoni* infection on the smooth muscle function might be indirect through alterations of the myenteric plexus. The interstitial cells of Cajal (ICC) are mesenchymal cells arranged in a characteristic manner in close relation to the nerves and smooth muscle cells in the outer muscular coat of the GI tract. There is now direct evidence that the ICC act as pacemakers in specified regions of the gut in some species. The best evidence pertains to the ICC surrounding the myenteric plexus (ICC-MP) of the mouse stomach and small intestine. The tyrosine kinase receptor kit is required for the development of ICC and of slow-wave activity in the mouse small intestine. Kit immunoreactivity (Kit-ir) is now widely used to identify ICC by immunohistochemistry in experimental animals and in humans.

In the intestine, Kit-ir ICC forms a network in the myenteric plexus region (ICC-MP) that surrounds the myenteric ganglia. In the inner part of the circular muscle layer, another group of ICC, which expresses significantly lower levels of Kit-ir than the ICC-MP, forms a distinct network corresponding to the deep muscular plexus (DMP). Double labeling with c-kit and markers for neuronal elements indicated that the network of ICC surrounds the ganglia and nerve bundles of the myenteric plexus without entering them. Kit-ir ICC present a variable number of gap junction, identified by Connexin 43-ir, either between adjacent ICC or between ICC and myocytes. Moreover Connexin 43-ir gap junctions are abundant between myocytes in the circular muscle layer but not in the longitudinal muscle layer.

Abnormal distribution of ICC has been reported in several diseases and abnormal function of ICC might actually be involved in many disorders of GI transit. Faussone-Pellegrini *et al.* have reported alterations of ICC during and after inflammation induced by *Nippostrongylus brasiliensis* in the rat. At day 30 post-infection with peaked inflammation, a complete loss of ICC-DMP in the jejunum was observed, causing alterations in motility.

Since intestinal schistosomiasis involves disturbed gut motility, the aim of the present study was to delineate if ICC were similarly affected during *S. mansoni* infections. A murine model of *S. mansoni*–outbred Swiss mice was used to investigate the distribution of ICC in relation to that of the muscular and neuronal elements in the mouse ileum, during acute and chronic stages of infection.

**Material & Methods**

*Schistosoma mansoni* infection: The maintenance of the *S. mansoni* life-cycle and the transcutaneous infection of mice with *S. mansoni* have been previously described. Male Swiss mice (age 7 wk) were anaesthetised with Nembutal® (60 mg/kg) and the abdomen was shaved. A metal ring was placed on the abdomen and then filled with treated water containing 70 infectious cercariae of a Puerto Rican strain of *S. mansoni*. The cercariae were allowed to penetrate during 20 min after which the water was removed and checked for remaining cercariae. Groups of 10 infected mice were sacrificed after 8 and 15 wk, together with age matched uninfected controls. The ileum was
dissected out and transferred to a Petri dish filled with Hanks calcium free solution at pH 7.2 and 37°C. Mesentery and fat were carefully removed without damaging the serosa.

Research protocols involving rodents received ethical clearance by the University of Antwerp ethical committee.

Sections: Ileum segments, approximately 1 cm long, were fixed in fresh 4% paraformaldehyde solution in PBS, pH 7.4, at 4°C for 4 h, cryopreserved in sucrose solutions, embedded in Tissue-tek OCT compound (Miles, Elkhart, IN), orientated, snap-frozen and stored at –80°C. Sections (10 μm thick) were cut on a cryostat, mounted on slides coated with 0.1% poly-L-lysine (Sigma, St. Louis, MI) and stored at –20°C until use.

Whole mounts: Specimens were opened along the mesentery, the contents cleaned and pinned flat, mucosa facing down, on the bottom of a petri dish coated with Sylgard elastomer (Dow Corning Co., Midland, MI). After several rinses in PBS, specimens were fixed in fresh 4% paraformaldehyde solution in PBS, pH 7.4, at 4°C for 3 h. After three rinses in PBS, the mucosa was carefully peeled off under a stereomicroscope. Whole-mounts were stored in PBS containing 0.1% sodium azide at 4°C and used within a week. A minimum of two whole-mount preparations from two different animals, were used for each level studied.

Antibodies: Primary antibodies used were goat anti-serum raised to mouse tyrosine kinase receptor Kit (Santa Cruz Biotechnology, Santa Cruz, CA, dilution 1/900), rabbit antiserum to protein gene product 9.5 (PGP-9.5, Ultraclone, Isle of Wight, UK, dilution 1/3000), rabbit antiserum to the small conductance calcium activated potassium channel SK3, a marker for Kit negative fibroblast-like cells (FLC)8 (Alomone Labs, Jerusalem, Israel dilution 1/300) and rabbit antiserum to Connexin 43 (Cx43, Chemicon, Temecula, CA, dilution 1/100). Secondary antibodies used were: donkey anti-goat IgG coupled with fluorescein (FITC), and donkey anti-rabbit IgG either coupled with FITC (for single labeling) or coupled with Texas Red (for double labeling), at a dilution of 1/200 (Jackson Immunoresearch Laboratories, West Grove, PA).

Double immunofluorescence staining: Double immunofluorescence staining was performed as described11. Briefly, slides were brought to room temperature (RT) rinsed in 10 mM TRIS (Merck-Belgolabo, Overijse, Belgium) and 0.15 M sodium chloride, pH 7.4 (TRIS-buffered saline, TBS), containing 0.1% (v/v) Triton X-100 (TBS-TX), and incubated for 1 h in 10% normal horse serum (NHS) (Hormonologie Laboratoire, Marloie, Belgium) and TBS-TX to reduce background staining. The slides were left overnight at RT in a humid chamber with the primary antibodies diluted in TBS-TX containing 2% NHS, rinsed in TBS and then incubated in the dark for 1 h at RT in TBS containing the donkey anti-rabbit Texas Red and donkey anti-goat FITC secondary antibodies.

Confocal microscopy: After five rinses in TBS, coverslips were mounted with ‘slow fade light’ anti-fade mounting medium (Molecular probes) in 50% glycerol and secured with nail polish before viewing under a confocal microscope (MRC 1024; Bio-Rad Laboratories, Hemel Hempstead, Herts, UK) fitted on an inverted microscope (Axiovert 100; Zeiss, Oberkochen, Germany) equipped with Plan-Neofluar ×40/1.3 and ×63/1.4 oil immersion objectives (Zeiss). The excitation beams of an argon/krypton laser (488 and 568 nm) and band-pass emission filters (522/35 and 605/32 nm) were sequentially used for selective detection of the green (FITC) and red (Texas red) fluorochromes respectively.

Optical sections, through regions of interest in each preparation (X-Y: 768 × 512 pixels), 1 μm apart (Z-step), were collected sequentially for each fluorochrome. The three gray-scale data sets generated were
transferred to a Indy workstation (Silicon Graphics, Mountain View, CA) running ImageSpace software (Molecular Dynamics, Sunnydale, CA). The two data sets were merged to form a ‘Z-serie’ and displayed in green and red for FITC and Texas red respectively. Single-colour and merged images corresponding to single optical sections were individually reviewed.

For rendering the strong autofluorescence of the inflammatory infiltrate present in infected animals, in combination with the immunofluorescent signal, the red and green dataset were digitally combined and after thresholding, pixels identified in both the green and the red lines (further referred to as “the autofluorescence”), were arbitrarily displayed in green, while the specific Texas Red immunofluorescent signal was displayed in red. Pictures were exported on a Power Mac (Apple, Cupertino, CA) running Freehand (Macromedia, San Francisco, CA) and Illustrator (Adobe, San Francisco, CA) software.

Results

Inflammation caused by S. mansoni egg stage parasite: Infection with S. mansoni causes the parasite egg stages to lodge in the murine ileal wall inducing focal granulomatous responses. This process leads to inflammation and muscular hypertrophy within the ileal wall. At 8 wk post-infection, parasite eggs are lodged in the ileal wall, inducing a granulomatous response that expands in size to form diffuse infiltrates in the ileal muscle wall at 16 wk post-infection (Fig.1).

Distribution of ICC in diseased status in relation to the nervous and muscular systems: A lack of gross alteration in the distribution of PGP 9.5-ir enteric nervous system in uninfected and infected ileum was observed (Fig. 2). Kit immunoreactivity is now widely used to identify ICC by immunohistochemistry (referred to here as Kit-ir ICC). The distribution of Kit-ir ICC in the ileal muscle layers in relation to the nervous system (as stained by PGP 9.5 immunoreactivity), demonstrated the absence of changes in the distribution of KIT-ir ICC in the diseased status (Figs. 3 & 4). There was no indication of the disappearance of either the ICC-MP or ICC-DMP, or for any decrease of these layers with acute or chronic infection on both whole mounts and sections. Cx43-ir gap-junction proteins were identified in the circular muscle layer of uninfected and infected ileum. With the onset of inflammation in the infected status, hypertrophy of the ileal musculature was observed, yet, even in the chronic stages of infection, the Cx43-ir was abundantly present in the circular muscle layer and absent from the longitudinal muscle layer, as in uninfected controls (Fig. 5).

Distribution of non-Kit fibroblast like cells in the ileum: SK3-ir FLC apparently increased with advanced infection (Fig. 6). Noteworthy, the dense network of SK3-ir FLC was found in close proximity to inflammatory cells, presumably macrophages, exhibiting a very strong autofluorescence (Fig. 6).

Discussion

Experimental models of granulomatous inflammation like schistosomiasis permit detailed analysis of the basic immune mechanisms involved in chronic immunoregulation. In schistosomiasis, granulomas are the consequence of an immune response to helminthic ova that are deposited in the host’s tissues. The purpose of this granulomatous response is to sequester the ova and their harmful secretions, eventually killing the larva. The granulomas, however, are the main pathogenic agent in schistosomiasis, being space-occupying structures that evolve in fibrotic scar tissue. Complex immunoregulatory circuits govern the granulomas.

Murine schistosomiasis is a particularly useful model for studying in detail features of inflammation. S. mansoni causes both acute and chronic disease with a wide range of symptoms. In the acute phase intestinal manifestations like abdominal pain, disturbed GI
Fig. 1: Inflammation and muscular hypertrophy of the ileal wall caused by *S. mansoni* eggs. Hematoxylin and eosin (H&E) stained transverse sections of control, uninfected, mouse ileum section (Fig. 1a); 8 wk after infection with *S. mansoni* (Fig. 1b); and 16 wk after infection (Fig. 1c). Magnification 40x.

Fig. 2: Neuronal network in an uninfected (Fig. 2a) and 8 wk infected (Fig. 2b) ileum whole mount stained by PGP 9.5-ir (neuronal marker for protein gene product 9.5). Maximum intensity projections of 15 micron thick stacks. Scale bars: 20 microns

Fig. 3: The relationship between PGP 9.5-ir neural elements (in red) and KIT-ir interstitial cells of Cajal (in green) in an uninfected whole mount, at the level of the myenteric plexus region (Fig. 3a) and at the level of the deep muscular plexus (Fig. 3b). Maximum intensity projections of 15 and 8 micron thick stacks, in 3a & 3b, respectively. Scale bars: 20 microns

Fig. 4: Similar relationship between PGP 9.5-ir neural elements (in red) and the ICC identified by immunoreactivity for the Kit tyrosine kinase receptor (in green) on a whole mount of an acute stage (8 wk) infected mice ileum (Fig. 4a); and of a chronic stage (15 wk) infected mice ileum, in the myenteric plexus region (Fig. 4b) and deep muscular plexus region (Fig. 4c) of the same whole mount. The bright orange punctate labeling labels strongly autofluorescent inflammatory cells, which were fairly abundant in chronic stages of inflammation. Maximum intensity projections of 15 micron thick stacks. Scale bars: 20 microns
contractility leading to diarrhea, nausea and fever occur. The main impact on public health is due to the chronic infection leading to severe intestinal involvement resulting in bloody diarrhea. It is this intestinal involvement of *S. mansoni* infection that is most important in terms of public health. *S. mansoni* egg-caused inflammatory reactions in the gut epithelium alter GI motility in humans and in experimental animal models. The mouse is a fully susceptible host to *S. mansoni* and can be used to study the effects of *S. mansoni* infection on GI muscle contractility.

The role of the myenteric plexus in the *Schistosoma*-induced alterations of GI motility remains an enigma. Morphological alterations of the myenteric plexus have been described, and myenteric plexus destruction is said to lead to an increase in intestinal muscle thickness. Enteric nerves may play a role in the initiation and maintenance of intestinal inflammation but at a later-stage inflammation may also alter the normal function of enteric nerves. Patients with inflammatory bowel disease show nerve fiber hypertrophy and architectural alterations in the nerve plexuses and nerve cell bodies in the enteric nervous system. There is also increasing, electrophysiological evidence that ICC may be actively involved in particularly inhibitory, nerve transmission in the GI tract.

The recognition of the ICC as important elements in the regulation of GI motility has opened new perspectives for a better understanding of GI motility in human disease status, which have been emphasised in recent reviews. The ICC by forming networks in the gut wall may play an important role in the control of digestive motility, by generating slow wave activity of the gut musculature. Absence or reduced numbers of Kit-ir ICC have been reported in an increasing number of conditions including infantile hypertrophic pyloric stenosis, Hirschsprung’s disease. Furthermore, ultrastructural alterations specifically affecting the ICC have been reported in ulcerative...
colitis. Faussone-Pellegrini et al. have reported a loss of ICC-DMP in the jejunum after inflammation induced by Nippostrongylus brasiliensis in rat that alters GI motility.

In the case of schistosomiasis all evidence in our experiments pointed to the presence of both the ICC-MP and ICC-DMP in diseased ileum tissues. The PGP stained innervations appear the same with infection, SK3 stained cells, however, increased with advanced infection. Moreover, this dense staining was found in close proximity to autofluorescent inflammatory cells.

Our study thus delineates that the ICC are not lost during S. mansoni infections, and thereby structural defects of the ICC networks are likely not responsible for mediating the disturbed GI motility patterns caused by schistosomiasis. Both whole mounts and sections depict that muscle thickness and inflammatory infiltrate increased with infection while the distribution of Cx43-ir gap junctions remained unaltered. Kit-ir ICC and SK3-ir FLC were present at all normal locations and during acute (after 8 wk) and chronic (after 15 wk) stages of infection as seen in controls. No disappearance of either ICC-MP or ICC-DMP populations was noted. A preferential, although not exclusive, location of inflammatory infiltrate in close vicinity with SK3-ir FLC in the muscle layer was observed.

In recent years, evidence has accumulated to support the view that ICC play important roles in GI motility. However, it might be misleading to specify this activity generally with regard to all inflammatory diseases. Data currently available are still very limited, thus more evidence is required before the ICC can be confirmed to be involved in pathophysiological processes of GI disorders.

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