CORRELATION BETWEEN SMALL INTESTINE ENTEROCHROMAFFIN CELLS AND MIGRATING AND EXCRETED SCHISTOSOME EGGS DURING EXPERIMENTAL *SCHISTOSOMA BOVIS* INFECTION IN CATTLE.

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SUMMARY

In schistosomiasis, the mechanisms of extravasation and migration of schistosome eggs into the intestinal lumen, and diarrhoea are not very well understood. In addition, the role of the neuroendocrine system has not been investigated. Therefore, Schimorl's ferricyanide stain and immunohistochemical staining for 5-hydroxytryptamine- like immunoreactivity were used to study enterochromaffin cells in the jejunum and ileum of 12, one year old, Jersey and Friesian Boran crossbred male calves during experimental *Schistosoma bovis* infections (6 calves) to elucidate their role in the pathophysiology of migration and egress of *Schistosoma bovis* eggs and diarrhoea. Most of the enterochromaffin cells reached the intestinal cryptal lumina. 5-hydroxytryptamine immunoreactive granules in the enterochromaffin cells varied in size and were in most cells dispersed in the entire cytoplasm and in addition, some reactivity could be traced in the uppermost parts and in the cryptal lumina. Smaller granules appeared in the luminal part of the cytoplasm, while the larger and more intensely stained granules were localized in the basal compartment. Between weeks 9 - 13 post infection, when diarrhoea was recorded in two infected calves, the excretion of eggs was at maximum and the numbers of enterochromaffin cells in infected calves increased to become significantly higher than those in control calves during weeks 11 - 13. The numbers of enterochromaffin cells in infected calves were

positively correlated to the number of schistosome eggs per gram of faeces. The results are discussed with respect to the mechanisms of migration and egress of schistosome eggs from intestinal wall outside the host body and diarrhoea.

Key words: Enterochromaffin cells, *Schistosoma bovis*, small intestine, cattle.

INTRODUCTION

Schistosoma bovis and *Schistosoma mattheei* are the major cause of schistosomiasis in livestock in Africa (Dinnik and Dinnik, 1965). In Tanzania, *S. bovis* is reported to be a dominant species (Kassuku *et al.*, 1986). Losses due to livestock schistosomiasis include mortalities, retardation of growth, poor reproductive performances and condemnations of organs at slaughter (Saad *et al.*, 1980; McCauley *et al.*, 1983).

Major clinical and pathological changes observed in intestinal schistosomiasis are due to the host defence reaction to migrating of eggs in the intestinal wall, eggs trapped in the intestinal wall as well as live and dead adult worms in the mesenteric and intestinal veins (Hussein, 1971, Hussein *et al.* 1975; Lawrence, 1978; Saad *et al* 1980). During early *S. bovis* infections in cattle the small intestine is the most affected (Hussein, 1971; Hussein *et al.* 1975). Mature schistosomes lay eggs in the venules deep in the intestinal wall and the mesenteric veins (Lawrence, 1978). Thereafter, eggs in the intestinal wall migrate to exit to the intestinal lumen and are shed by faeces. Extravasation, migration and egress of schistosome eggs into the intestinal lumen are speculated to be facilitated by endothelial cells, fibroblasts and inflammatory cells (Lenzi *et al.*, 1987; Semuguruka 1992) as well as lytic excretions from the miracidia in eggs and leukocytes (Kuba, 1963; Semuguruka, 1992). The process is also thought to be facilitated by peristalsis (Kuba, 1963) and immune complexes around the egg (Doenhoff *et al.*, 1986).

Diarrhoea is one of the clinical manifestations of *S. bovis* infections in cattle (Hussein *et al.*, 1975; Saad *et al.*, 1980; McCauley *et*

al., 1983; Semuguruka, 1992). It is considered to be due to physical damage to intestinal venules and mucosal epithelium by exiting eggs (Lawrence 1978; Saad *et al.*, 1980; Semuguruka 1992) and inflammatory reactions due to migrating and trapped eggs as well as other factors (Semuguruka (1992).

The enteric nervous system (ENS) and the neuroendocrine cells play a major role in stimulating and regulating the mechanisms of secretory diarrhoea via highly integrated and complex mechanisms. It is hypothesized that 5-Hydroxytryptamine (5-HT), substance P and neurokinin from epithelial mucosal cells activate the ENS plexuses which in turn release acetylcholine, vasoactive intestinal polypeptide (VIP) and other endogenous effectors in the effector pathways to stimulate chloride secretion (Hansen and Skadhauge 1995; Hansen and Skadhauge, 1997). The intracellular mediators for 5-HT include eicosanoids, calcium, phosphoinositols and may be nitric oxide (NO) and cylic nucleotides (Hansen and Skadhauge, 1997).

Inflammatory cells such as neutrophils, lymphocytes (Wallis *et al.*, 1990) and mast cells and prostaglandins (Pothoulakis *et al.*, 1998) play a role in inducing fluid secretion. It is hypothesized that diarrhoea due to infectious agents results from colonization, adherence and or epithelial invasion which is followed by release of cytokines from enterocytes which activates resident phagocytes and recruitment of new phagocytes in the lamina propria and activation of the ENS (Powel (1994)). On the other hand, diarrhoea due to nematode infestation is speculated to be due to intestinal anaphylaxis induced by mast cell activation (Powel, 1994). In these two hypotheses, it has been proposed that inflammatory mediators from mast cells and phagocytes cause intestinal chloride and water secretion as well as inhibition of neutral sodium and chloride absorption. These events occur by direct effects of the mediators on the epithelium, release of prostaglandins from lamina proprial fibroblasts and activation of the enteric nervous system (Powel, 1994). This phenomenon is however not well investigated in trematode infestations.

In murine *Schistosoma mansoni* infections, neurones close to granulomas exhibit increased staining for VIP (Varilek *et al.*, 1991) and granuloma eosinophils synthesize and produce VIP and Substance P (Weinstock, 1991). Apparently, the complex interactions of chemical mediators from neuroendocrine and inflammatory cells, and the ENS may offer a new paradigm for the mechanisms facilitating the migration and egress of schistosome eggs and which cause diarrhoea in schistosomiasis.

In enteropathy of coeliac disease (Sjolund *et al.*, 1982; Challacombe and Robertson, 1997) the duodenal mucosa was charactarized by hyperplasia and hyperactivity of EC cells. A similar observation was recorded in the small intestine of mice infected with the tape worm *Hymenolepis diminuta* leading to a proposition that EC cells and the ENS are involved in the mechanisms of cestode rejection (McKay *et al.* 1990).

In the small intestine, 5-HT is localized in EC cells, mast cells and the ENS with the EC cells possessing the highest concentration of 5-HT (Ormsbee and Fondacaro, 1985). EC cells also have been shown to contain motilin (Krstic, 1984), substance P (Heitz *et al.*, 1976), enkephalins (Heitz *et al.*, 1978) and guanylin (Cetin *et al.*, 1994). In the intestine of cattle, EC cells are most numerous in the duodenum and in the large intestine (Kitamura *et al.*, 1985). They appear first during the third month of gestation (Totzauer, 1991). Responses of EC cells to secrete chemical mediators are facilitated by microvilli tufts situated on the luminal surface of the open type of EC cells, which monitor changes in the environment in the lumen of the gastrointestinal tract (GIT) (Cetin, *et al.*, 1994). On the interstitial side, release of chemical mediators is effected by receptor mediated mechanisms via stimulation of beta-adrenoceptors, muscarine, nicotine and 5-HT3 receptors (Racke *et al.*, 1996). The aim of this study was to elucidate if migrating and exiting eggs, and excretions from the miracidia within the eggs can stimulate EC cells.

MATERIALS & METHODS

Animals, infective cercariae, infection and *S. bovis* eggs

One year male calves (N = 12) of Jersey- and Friesian-Boran cross breed were used in the experiment. The calves were kept outdoors, grazed on natural pastures and supplemented with maize bran. During the first two weeks calves were screened for helminth eggs and blood parasites. Thereafter, calves were randomly allocated into the control and experimental groups, six in each. Calves in the experimental group (N = 6) were exposed to infective cercariae per cutaneously as described by Semuguruka (1992) by giving each calf approximately 10,500 cercariae. The *S. bovis* cercariae were obtained from the Department of Veterinary Microbiology and Parasitology - Sokoine University. Faecal samples were taken from all calves at two weeks intervals for counting eggs per gram of faeces (epgf) by using the Modified Bell (1963) filtration technique described by Kassuku *et al.* (1985).

Sampling, processing for paraffin sections, staining and EC cell count

From each group, one calf was randomly selected and euthanised at weeks 7, 9, 11, 13, 17, and week 20 post infection (p.i) respectively. Within 20 - 25 minutes after death the abdomen was opened to expose the stomachs and intestine and two tissue segments about 3cm long each were collected from the proximal, middle and distal parts of both jejunum and ileum. Each tissue was cut into two smaller pieces of about the same size and opened along the mesenteric attachment. The tissues were immediately washed in 0.01M phosphate buffered saline (PBS) solution of pH 7.3, pinned on polystyrene squares and fixed in 4.5% buffered formaldehyde at 4°C. Tissues for microdissection were stretched during pinning and were fixed for 48 hours. Tissues for embedding in paraffin were not stretched during pinning and were fixed for 7 days and thereafter stored in 70% ethanol at 4°C. After one hour in fixative the pins were removed, tissues taken away from plastics and trimmed to approximately 2 cm long pieces for microdissection and 0.5 x 1 cm^2 pieces for embedding in paraffin.

Paraffin tissue blocks were sectioned by using a rotary microtome. Ten serial sections of 5mm thickness each were cut for

each of the intestinal site sampled and stained by Schimorl's ferricyanide stain. To count EC cells in these sections, a one millimetre square window was cut through a 1 x 2 cm^2 plastic paper (Letraset, England) which was glued over the cover slip after selecting an area with mucosa villi and crypts. By using an eyepiece calibrated into two square units by two perpendicular rulers, each field was divided into 4 equal areas. The numbers of EC cells were systematically counted in the four areas at x20 objective (magnification) after which the field was changed. When counting in the 1mm^2 window was over, the total number was recorded. Results were analysed statistically for each slaughter date and at the end of slaughtering by using the analysis of variance: random effects model (Synedcor and Cochran, 1989).

Microdissection and immunohistochemical staining

Microdissection to obtain mucosal wholemounts and subsequent localization of 5-HT-like immunoreactivity using the two step indirect streptavidin-ABComplex/HRP immunoenzymatic staining was done as described by Balemba et al. (1998). For detailed cellular morphology, wholemounts which were well stained were embedded in historesin (Leica, Heidelberger Germany) and sectioned at 2.5mm (LKB-Historange microtome, Cambridge instruments GmbH - Germany) and counterstained lightly by haematoxylin and eosin (H&E). All tissues were mounted in DPX and evaluated by light microscopy.

RESULTS

The calves showed no signs of intestinal helminth infection and blood parasites at pre-experimental screening. Clinically, infected calves showed anorexia, loss of body condition and abdominal pains. Two infected calves manifested overt diarrhoea during weeks 11 to 13 post-infection (p.i).

Mucosal wholemounts which were stained for 5-HT and thereafter embedded in historesin, sectioned and counterstained by H & E revealed large triangular to polygonal shaped cells having large, basally located nuclei. Based

on 5-HT-like IR, we recorded: (a) Cells which had immunoreactive granules in the whole cytoplasm and which reached intestinal crypt lumina by narrow cytoplasmic extensions. These cells were predominating and their immunoreactive granules could be traced into the uppermost parts of the narrow cytoplasmic extensions adjacent to or into the crypt lumina **(Fig. 1a)**. (b) Cells in which positive staining granules were strictly localized in the basal part of the cytoplasm. The immunoreactive granules in both types of EC cells varied in sizes and staining intensities. Fewer and smaller granules appeared in the innermost part of the cytoplasm while, abundant, larger and intensely stained granules were localized basally close to basal lamina. Enterochromaffin (EC) cells were abundant in the deep part of the crypts with a decreasing number along the crypt- villi axis so that they became few at the tips of the villi **(Fig. 1b)**.

The number of EC cells varied significantly between control and infected calves at all slaughter dates being most pronounced during weeks 9 to 13 p.i. **(Fig. 2)**. In the jejunum and ileum of both control and infected calves, variation between sites was highly significant ($P < 0.001$) (not shown). The number of enterochromaffin cells in the jejunum decreased in the aboral direction whereas in the ileum they increased the in aboral direction (not shown). Generally the number of EC cells in the jejunum was greater than in the ileum as EC cells in the jejunum were significantly greater ($P < 0.001$) than in the ileum in both infected and control calves during weeks 7, 9, 13 and 20 p.i.

Between weeks 9 and 13 p.i, there was a marked increase in the number of EC cells in both the jejunum and ileum of infected calves compared to the control calves. During this period the number of EC cells in the jejunum and ileum of control calves decreased. Comparing the number of EC cells in the jejunum and ileum for both groups, the numbers of EC cells in infected calves were significantly higher ($P < 0.001$) than in the control calves during weeks 11 and 13 P.i.

Fig. 1a Historesin section (2.5 μm) from the submucosal wholemount which was initially stained for 5-HT-like IR thereafter embedded in historesin, sectioned and counterstained by H & E. Note: two enterochromaffin cells each with a large nucleus (N) located basally. The "open type" of enterochromaffin cell has a narrow apical cytoplasm (arrows) which reaches the lumen of crypt (Cl). IR granules for 5-HT are not seen. X 4,680. Fig.

1b A 5 μm paraffin section stained by Schimorl's ferricyanide from the middle ileum of the control calf, week 17 p.i. Note: enterochromaffin cells (arrows) in the crypts of the mucosa (Cl). Lamina muscularis mucosae (L), tela submucosa (Sm) and the nner circular muscle (Ic) are also seen. X 1,520.

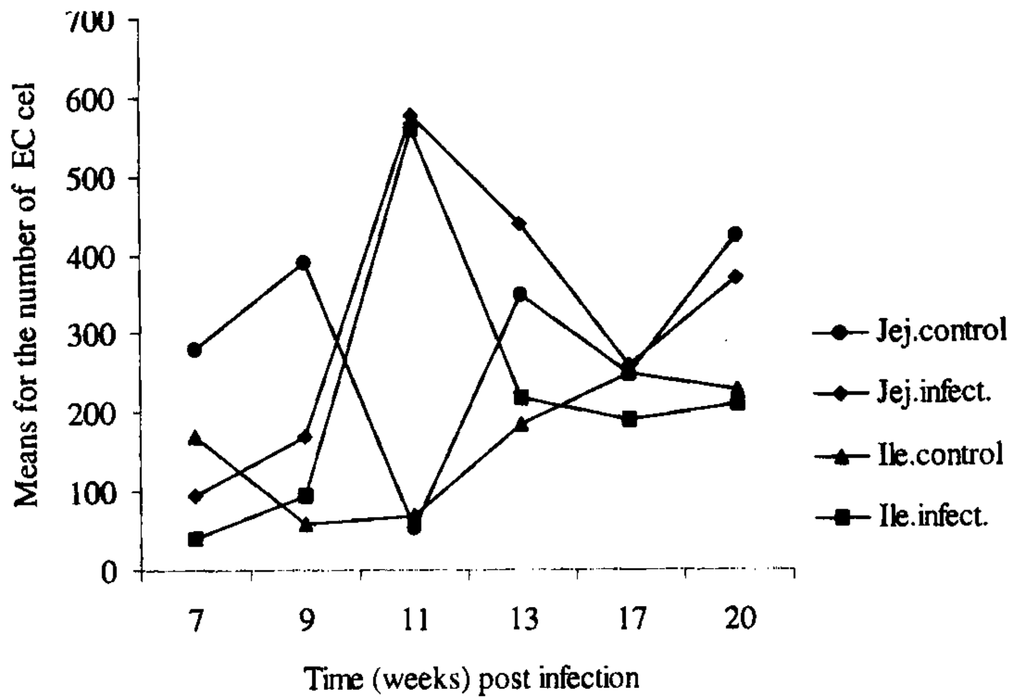

Fig.2. Means for the number of EC cells in the jejunum and ileum of calves infected by *S.bovis* and controls

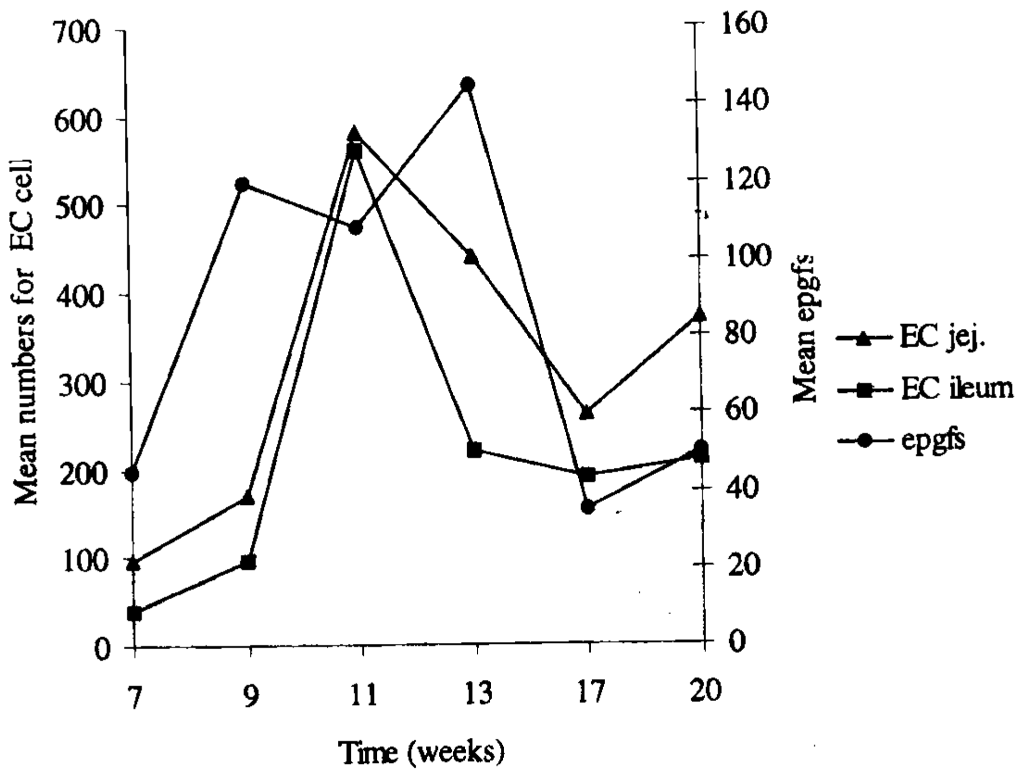

Fig. 3. Means of the number of EC cells in the jejunum and ileum and epgfs of S. bovis infected calves

However, the number of EC cells in the control calves was significantly greater (P < 0.001) than that of infected calves at weeks 7, 9, 17 and 20 p.i. *S.bovis* were detected first on day 41 p.i. (end of week 6 p.i.). There was an increase in epgf from week 7 p.i. *S.bovis* were detected first on day 41 p.i. (end of week 6 p.i.). There was an increase in epgf from week 7 p.i. and higher epgf values were observed during weeks 9, 11 and 13 p.i. after which there was a marked decrease in epgf. The number of EC cells in infected calves was positively correlated to the number of schistosome eggs per gram of faeces (epgf) (Fig. 3). An increase in epgf was associated by a corresponding increase in the number of EC cell. Similarly, the decrease in epgf was associated with a decrease in the number of EC cells.

DISCUSSION

The observations of the onset of excretion of *S. bovis* eggs in faeces at day 41 (5½ weeks) p.i and clinical signs and diarrhoea during weeks 11 to 13 p.i support the findings by Hussein (1971) that clinical signs are related to oviposition and excretion of eggs though the intestinal mucosa and most obvious when excretion of eggs in faeces is at maximum. Observation of diarrhoea, in two calves out of six, support the findings by Saad *et al.*, (1980) that the severity of the signs depends on infection intensity, host susceptibility, duration of infection and nutritional status of the animal.

The finding that most of the EC cells reached intestinal lumen by narrow cytoplasmic extensions suggest that the majority of EC cells were of the "open" type. These findings are in accordance to those of Kobayashi *et al.*, (1970), Cetin *et al.*, (1994) and Totzauer (1991) that the majority of EC cells are bipolar. In the present study, 5-HT-like IR granules in EC cells could be followed up into the luminal parts of the cytoplasm and the crypt lumina. This observation supports the hypothesis of continuous endoluminal secretion of 5-HT ("spilling over") both in normal and pathological states (Nilsson *et al.*, 1987; Schworer and Racke, 1991). This observation also

supports the hypothesis of the trophic effect of 5-HT in stimulating the production rate of the crypt cells proposed by Wheeler and Challacombe (1987). The observation of many, large and intense 5-HT-like IR granules in the basal compartment supports the report of Racke *et al.* (1996) that secretion of 5-HT from EC cells occurs predominantly at the interstitial side.

The findings on the distribution of 5-HT IR granules are consistent to those of Nilsson *et al.* (1987). In the present study, 5-HT IR granules varied in sizes. The reason for this variation could be due to that granules were at different stages of maturation in the Golgi complex or varied in the co-packed secretory constituents as 5-HT is colocalized with motilin (Krstic' 1984), substance P (Heitz *et al.*, 1976), enkephalins (Heitz *et al.*, 1978) and guanylin (Cetin *et al.*, 1994). It could also be an indication of different forms of storages of 5-HT advocated by Nilson *et al.* (1987) or an indication of granules at different stages of development.

The finding that EC cells in the jejunum decreased aborally in contrast to an aborally increase in the ileum are consistent to those of Kitamura *et al.*, (1985); Cho and Nabuo (1988) in cattle. The significant variation in the number of EC cells along sites supports similar observation by Kitamura *et al.*, (1985).

In the present study, the number of EC cells in infected calves was significantly higher than in control calves especially between weeks 9 and 13 p.i. The highest cells count for both jejunum and ileum was recorded in the infected calf at week 11 p.i. During this period we recorded higher values of epgf and the number of EC cells and epgf were positively correlated. In addition, diarrhoea was recorded in two calves as stated earlier. The period when high number of EC cells, high epgfs and diarrhoea were recorded is in agreement to the period when high numbers of extravascular *S. bovis* eggs were recorded in the intestine of cattle (Semuguruka, 1992).

Seemingly, diarrhoea occurred when many eggs were exiting into intestinal lumen. Most probable, diarrhoea was due to, among other factors, the increased 5-HT activity in the EC cells following

an increase in the number EC cells and hence EC cells granulation index. The observations therefore, indicate the importance of EC cells and 5-HT in pathogenetic mechanisms of diarrhoea in *S. bovis* infections. They support the reports by Hansen and Skadhauge (1995, 1997) that 5-HT plays a major role in the development of secretory diarrhoea. They also support the hypothesis that diarrhoea due to helminth infestation is caused by intestinal chloride and water secretion as well as inhibition of neutral sodium and chloride absorption (Powel, 1994). Probably, the severity of diarrhoea in schistosomiasis is related to the number of migrating and exiting schistosome eggs, the number of EC cells and extent of EC cells and 5-HT activity.

5-HT stimulate vascular permeability, smooth muscle contractility and intestinal fluid secretion into the lumen which aid in dislodgement and expulsion of the worms (Tizard, 1982). In the intestine of the guinea pig, peristaltic reflexes which are regulated by the ENS depend upon activation of 5-HT transporter localized on the EC cells (Wade *et al.,* 1996). The increase in the number of EC cells between weeks 9 to 13 p.i., following an increase in excretion of schistosome eggs (weeks 7 to 13 p.i.), indicates that schistosome eggs migrating in tunica mucosa and those exiting into the intestinal lumen and/or excretions from miracidia in the eggs stimulate hyperplasia of EC cells and subsequent synthesis and release of 5-HT. On the other hand, high level of 5-HT may facilitate the migration of schistosome eggs and thereafter their explosion by faeces by stimulating intestinal motility. Therefore, our findings in *S. bovis* are similar to those of McKay et al. 1990 in the small intestine of mice infected with the tape worm *Hymenolepis diminuta* and gives further support to their hypothesis that EC cells an the ENS are involved in cestode rejection. In addition, our results showed that the extent of stimulation of EC cells was the same in both the jejunum and ileum. It may be speculated that the extent of proliferation of EC cells is related to the number of exiting and migrating eggs in the mucosa hence, correlated to the number of eggs in faeces. However, the exact

mechanism by which migrating and exiting *S. bovis* eggs and excretions from the miracidia might stimulate proliferation of EC cells needs to be investigated.

Between weeks 13 to 17 p.i., there was a decrease of epgf and the number of EC cells and cessation of diarrhoea. These findings may be explained by the fact that in *S. bovis* infections, animals develop immunity against schistosome eggs at around week 13 p.i. as a result, the number of eggs in faeces decreases during week 13 to 14 p.i. (Saad *et al.*, 1980). Additionally, this corresponds to the period in which worms are reported to shift from the small intestine to the colon (Saad *et al.*, 1980) and when few schistosome eggs have been observed in the small intestine (Semuguruka, 1992).

According to De Bruïne *et al.*, (1992), EC cells in the caecum of rat are renewed by the proliferation of prexisting cells and recruitment from precursor cells. 60-65% of the EC cells is the rapidly renewing and migrating cells with the turnover rate of about 16 days. The remaining percentage is the slow renewing group with the turnover time of about 150 days. However, in fasting rats (*Solomatina et al.*, 1985), an increase in the number of EC cells and intense argentaffin reaction were observed after 7 days. The turnover rate of EC cells in the small intestine of cattle is not known. Possibly, it may be close to that of the rat caecum. However, the present findings and those of Solomatina *et al.* (1985) indicate that there is a higher proliferative activity of EC cells in certain pathological states. The observation of an increase in the number of EC cell may be due to a higher mitotic activity in the crypts.

Celiac disease, ulcerative colitis, Crohn's disease and radiation enteritis are characterized by hyperpolasia of intestian EC cells (Dayal *et al.*, 1987; Challacombe and Robertson, 1997). The present investigation EC cells covered weeks 7 to 20 p.i. which is the acute and subacute period of schistosomiasis. It is not certain whether the hyperplasia of EC cells occurs in chronic infections or in repeated exposures that occur in endemic areas. During chronic *S. bovis* infection, fibroblasts in submucosa layer proliferate and

cause fibrosis and thickening of the submucosa layer (Hussein *et al.*, 1975; Semuguruka, 1992). The acidic fibrobalst growth factor (aFGF) which is localized in EC cells of normal GIT mucosa and EC cell tumors has been suggested oto stimulate proliferation and activity of stromal fibroblasts (La-Rosa *et al.*, 1997). The present findings of an increased number of EC cells in the small intestine of calves infected by *S. bovis* suggests that fibrosis and thickening of the submucosa layer may be due to an increase in aFGF derived from the higher number of EC cells.

CONCLUSIONS

The findings on morphology of EC cells and 5HT granules correspond to previous data in cattle and other species. However, the present study, has revealed that:

The number of EC cells in the small intestine of calves infected by *S. bovis* is significantly greater than that of control calves between weeks 9 and 13 p.i,. During this period, the egg per gram of faeces from infected are also high and positively correlated with the numbers of EC cells in the same calves, and some calves may develop diarrhoea. Most likely, the numbers of EC cells is also correlated to the number of migrating and exiting eggs in the intestinal mucosa.

The rate of proliferation EC cells during S. bovis infection in cattle is suggested to occurs more faster than the normal turnover rate (not shown).
The increase in the number of EC cells between weeks 9-13 p.i. following an increase in epgf during weeks 7 to 13 p.i. is associated with an increased granulation index of EC cells and thus increased 5-HT activity. Increases synthesis and release of 5-HT is supposed to subsequently stimulate hypersecretion and hypermotility via reflexes in the ENS nerves. These processes may facilitate the egress of migrating *S. bovis* eggs into the intestinal lumen and their excretion by faeces and contribute to the mechanisms for diarrhoea which is often seen in schistosomiasis.

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