

Response of Intestinal Mucosa to Trichinosis in immunized and non-immunized Albino Rat

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Abstract

The intestinal mucosa is reported to be greatly affected by infestation by *Trichinella spiralis*. The infestation by the worm is also controlled by intestinal tissue factors including cytokines and immune response. Vaccination with larval muscle antigen was reported to be of limited effect. However, vaccination with adult worm crude antigen may infer better protection. The aim of this work is to relate the histological, morphometric and immunological response of the intestinal mucosa of *T.spiralis* infested rat to worm burden, female fecundity and worm expulsion before and after immunization with crude antigen of the adult worm. This relationship is expected to give better evaluation of immunization by this antigen.

45 adult albino rats were allotted among three equal groups: control, *T.spiralis* infested and immunized infested group. Infection was done by 300 freshly isolated infective *T.spiralis* larvae. Five rats from each group were sacrificed at the end of the first, second and third week of infestation. The efficacy of immunization was assessed weekly by measuring adult worm burden and female fecundity, in relation to changes in the histological appearance, relative villus length, number of goblet, and mast cells and the amount of histamine in the intestine.

The results indicated that the worm burden was at its peak by the end of the first week after infestation. The mean protection value due to immunization in this parameter was 67%. Immunization resulted in a protection value relative to larval emergence of 38%. Maximum changes in the host tissue parameters were detected a week after that of worm burden. Measurement of the host intestinal response represented 78% change in villus/crypt ratio, 13.3% in number of goblet cells in the villus and 16.22 in the crypts, 22.5% in mast cell number and 16.8% in histamine content. The results indicate that immunization by crude antigen of the adult *T.spiralis* has a protective effect of the intestinal mucosa of the host due to inhibition of worm vitality and larval emergence. Mast cell production of histamine and goblet cell secretion of mucins could also play a role in protection.

Introduction

Trichinosis remains an important zoonotic food-borne parasitic disease of world wide distribution (Murrell, 2001). *Trichinella spiralis* parasitizes many mammals including man. The pig is considered the main component of the epidemiological chain (Leiby *et al.*, 1990).

Although in Egypt pork meat is not a common dish due to religious instructions, yet the risk of infection is still there, since sheep proved to be a source for human trichinosis (Rosa *et al.*, 1992; Kim, 1993; and Theodoropoulos *et al.*, 2000). Recently, *Trichonella* larvae have been also detected in ostriches (Piergilli-Foretti *et al.*, 2001) and horses (Viveros *et al.*, 2001). The

presence of *Trichinella* in these animals followed the use of proteins of animal origin in breeding herbivorous animals, which is now a common practice in many countries (Pozio *et al.*, 2001) including Egypt.

The relationship between parasites and hosts can be considered according to the concept that the host is an environment providing microhabitats for parasites to exploit (Rollinson and Anderson, 1985). Parasites divert resources from the host to their own use. So, the host is under strong selection pressure to stop, or at least, reduce this drain of his resources. One result of these pressures has been the evolution of

acquired immunity which can introduce changes in the environment of the parasite that are disadvantageous to it, and thus, help to restore the balance in favor of the host (Walkelin, 1993). There is a possible role of intestinal epithelial cells of the host in the protective immune response to *T.spiralis*. This role has been developed from observation of modified epithelial cell structure and function during a challenge infection. These changes included molecular and functional changes represented by reduced wheat germ lectin binding to the epithelial brush border membrane in infected rats (Bell, 1998), reduced hexose transport (Hessel et al., 1982), increased potential difference across epithelial tight junctions (Russell&Castro,1985), and chloride ion secretion, which is linked to a change from fluid uptake to net fluid secretion by enterocytes (Bell,1998). The functional changes in epithelial cells described in these studies have been linked to the immune system through immune serum transfer (Bell,1998). Immunity seems to play a role in expulsion of the adult worms of *T.spiralis* by the host. Although, it seems to be an ambiguous multi factorial process, but involvement of certain antibodies and cytokines has been well recognized lately (Bell,1998).

Although immunization has proven to be one of the most powerful weapons for prevention of infectious diseases, little prospect exists for the development of vaccines for parasitic infections (Gamble and Murrell, 1987). There is no doubt that the development of successful vaccination against human trichinosis is greatly needed. Prior infection with *T.spiralis* results in nearly complete immunity to challenge infection (Murrell, 1985). Antigens derived from different life cycle stages, likewise, can induce protective immunity (Gamble, 1985). However, it was found that the protection against challenge infection using antigens derived from the muscle of the larvae was partial (Murrell and Despomier, 1984 and Gamble.1985).

The aim of this work was to study the interaction between the extent of successful infection and the structure and activity of intestinal mucosa of rats infected by

T.spiralis worm alone or after immunization by crude antigen of the adult worm.

Material And Methods

Animals:

Forty five parasite free male inbred albino rats , 8-10 weeks old were divided into 3 equal groups: Control (C), parasite infected (P), and pre-immunized parasite infected (PI) group.

Parasite:

T.spiralis larvae were primarily obtained from infected pig diaphragm slaughtered at El-basateen abattoir, Cairo. They were maintained in the laboratory by serial passages in albino rats. Isolation of larvae from tissues was done according to Wassom *et al.*, (1983).

Antigen

Crude adult worm antigen (AA) was prepared according to the method of Goyal and Wakelin (1993). 7 day-old adult worms were collected from experimentally infected rats. The protein content of the worm homogenate was determined according to the method of Lowery *et al.* (1951). Homogenates were stored in 1ml vials stored at -20°C till use.

Protocol of Immunization and Infection

Rats of the immunized-infected group (PI) were immunized by two subcutaneous injections of 100µg AA emulsified in complete Freund's adjuvant (CFA, Sigma). The injections were one week apart. Rats of group (C) and (P) were injected in the same way by antigen free solution of CFA in phosphate buffer system (PBS). One week after the last injection, each animal from groups (P) and (PI) was infected with 300 freshly isolated infective larvae of *T.spiralis*. (Goyal and Wakelin, 1993).

Evaluation of the Efficacy of Immunization

Five rats from each group were sacrificed after ether anaesthesia by the end of the first, second and third week after parasite

infection. The small intestine was dissected out free of mesentery and subjected to the following examinations:

- A. Adult worm burden was performed according to the method described by Gamble (1985).
- B. Female worm fecundity was performed by *in vitro* release of larvae by female worms (Goyal and Wakelin, 1993). Fifty adult female worms were recovered on day 7 post-infection from 5 rats of each of the (P) and (PI) groups. The fifty worms of each group were incubated in universal tube containing 10 ml Hank's culture medium containing 10% fetal calf serum. The tubes then incubated for 4 hours at 37°C. The contents were filtered through two layers of gauze. The filtrate was transferred to centrifuge tubes. Few drops of formalin were added to each tube. The filtered culture was centrifuged for 10 mins at 1000rpm. The supernatant was decanted and the pellet was re-suspended in 1ml of PBS. The newly emerged larvae were counted in 10 aliquots of 10 μ l. The counts were expressed as (larvae/ female/24hrs).
- C. The percent protection was calculated in the immunized group according to Wang and Bell (1987).

Histological Studies

Histological preparations

A jejunum segment was dissected from the rats of each group, approximately 6 cm long. The segment was divided into 3 pieces each.

The first piece was opened, washed and fixed in Carnoy fluid for 24hrs. It was processed for paraffin embedding. 5 μ m sections were stained with toluidine blue for demonstration of mast cells.

The second piece was fixed in buffered formalin. It was then processed prepared for paraffin embedding. Serial 5 μ m sections were mounted on clean slides. Slide with one section was stained by hematoxylin and eosin (H&E) for general structure, and the next was stained by alcian

blue – PAS (AB-PAS) stain (Mowry, 1958) for demonstration of goblet cells and characterization of the type of mucin.

Quantitative Histology

Using H&E stained sections, 10 well oriented villi and crypts per rat were subjected to measurement of villus length and crypt depth using a calibrated micrometer mounted on Leitz microscope.

The alternative sections stained by AB-PAS were used to count the goblet cells in well oriented villi and crypts. The number of goblet cells per constant length using a Leitz microscope and eye piece graticule was recorded for 10 villi and crypts on each slide. Parts of the sections with well oriented villi and crypts with intact muscularis mucosa were chosen for counting goblet cells. Ten slides representing different animals of each group were counted.

Ten sections from the opened piece of jejunum stained with toluidine blue were used to count the number of mast cells per 10 constant areas from each slide. A Leitz microscope and eye piece graticule was used.

Histamine Content

The third piece of jejunum was used to estimate the amount of histamine in μ g/gm of intestinal tissue. The assay was performed according to the method of Lorenz et al.(1977) using the modified fluorometric assay.

Statistical Analysis

Data was statistically analyzed and graphically represented using Microsoft excel.

Results

Effect of Parasite on Intestinal Villi and Crypts

Histology and Histochemistry

In Hematoxylin and eosin stained sections, the intestinal mucosa of the control shows long finger like villi projecting in the lumen and crypts in between (Plate I: 1).

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After one week of infection with the parasite, the villi became short with blunt tips (Plate I: 2). The crypts appear relatively short and the muscle layer appears thickened. In parts of the sections, the worms appear invading the villi causing damage to the epithelial layer (Plate I, 2-insert). These changes are more prominent by the second week post infection (Plate I: 3)

In sections of the infected immunized group, the deteriorative changes appear less prominent than in the non-immunized group after one and two weeks of infection. (Plate I: 4, 5).

In sections stained by alcian blue-PAS, acidic as well as neutral mucin secreting goblet cells appear distributed along the villi and crypts of the control group (Plate II: 6). The intensity of the goblet cells is clearly high in non immunized group (Plate II: 7) and moderately high in immunized group (Plate II: 8).

In toluidine blue stained sections, mast cells are less in control (Plate III:9) than in treated groups (Plate III:10).

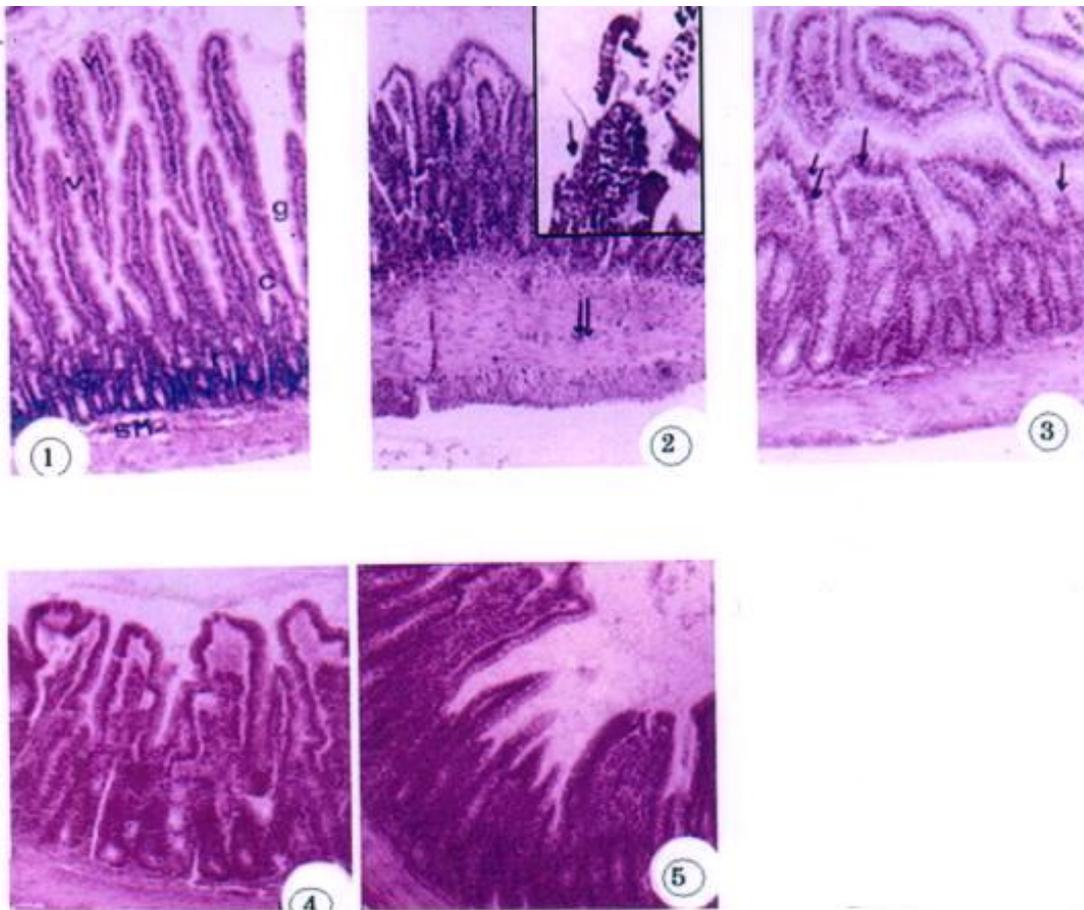


Plate I: Jejunal epithelium of 1) Control, 2) One week after worm infection: The insert represents the invasion of the epithelium by the adult worms., 3) Two weeks after infection, 4) Immunized infected group one week post infection, 5) Immunized infected two weeks post infection.

(HX, E, X100)

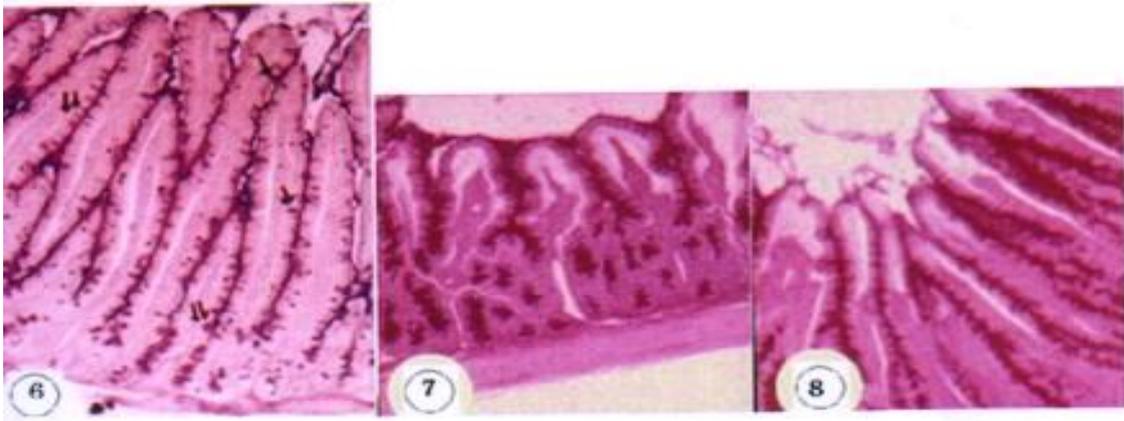


Plate II: Goblet cells in the villi and crypts of 6) Control, 7) infected animals two weeks post infection, 8) Immunized infected animals two weeks post infection.
(PAS, alcian blue X100)

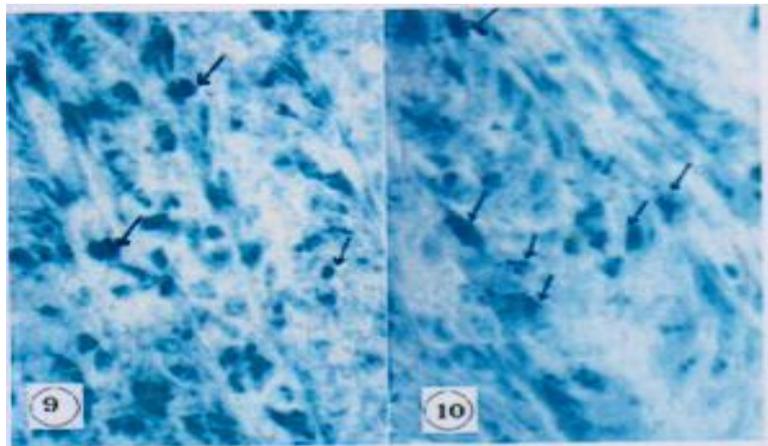


Plate III: Mast cells at two weeks post infection 9) non-immunized infected group 10) immunized-infected animals
(Toluidine blue X400)

Quantitative Evaluation:

Worm Burden

The change in worm burden after infection in non-immunized and immunized groups over the period of the experiment was used as a criterion for parasite infection. The worm burden at zero point was considered zero.

In figure 1-4, the worm burden has reached its peak value after one week. It started to decline over the next two weeks as the worms invade the mucosa. In the immunized group, there was 67% decrease in worm burden compared with the non-immunized group (table 1, figure 6).

Female worm Fecundity

The *in vitro* evaluation of the female worm fecundity indicated a 38% decrease in the number of larvae emerging from adults isolated from immunized compared to those isolated from non-immunized rats (table 1).

Villus/crypt Ratio:

In non-immunized group, there was a decrease in the mean ratio of the length of the villus relative to crypt length (figure 1). The least value was obtained on the second week after infection; after which a relative increase was obtained. The rate of the decrease in this ratio was 78% less after immunization.

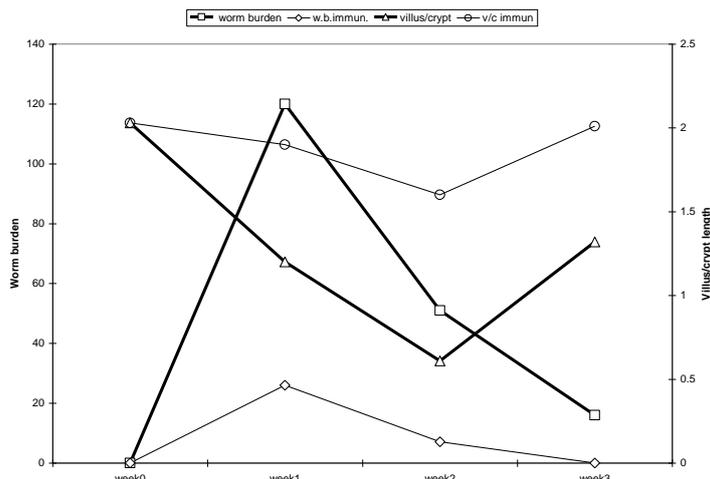


Figure (1): The change in the villus/crypt ratio in relation to the worm burden, over 3 week period, in rats infected by *T.spiralis* with or without immunization by adult worm antigen. The values for the control were considered as week zero. The villus crypt ratio is inversely proportional to the worm burden. The least value was obtained one week after the peak of the maximum worm burden.

The number of Goblet cells

Infection with *T.spiralis*, resulted in an increase in the number of goblet cells per unit area of the villar surface (table 2). The maximum value was obtained on the

second week, one week after the maximum worm burden (figure 2). In immunized group the increase in the number of goblet cells was 13.3% more than in the non-immunized group (table 1).

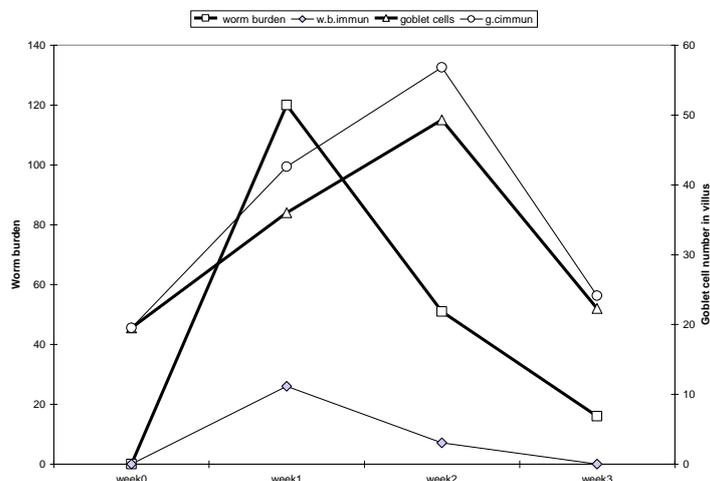


Figure (2): The change in the number of goblet cells in the villi in relation to the worm burden, over 3 week period, in rats infected by *T.spiralis* with or without immunization by adult worm antigen. The values for the control were considered as week zero. The villus goblet cell number is directly proportional to the worm burden. The highest value was obtained one week after the peak of the maximum worm burden.

The trend in the change in the number of goblet cells in the crypts was similar to that of the villi (table2,figure 3). However, the rate of increase after two weeks was

more in crypts than villi. The percent increase in the number of crypt goblet cells after immunization was 16.22%.

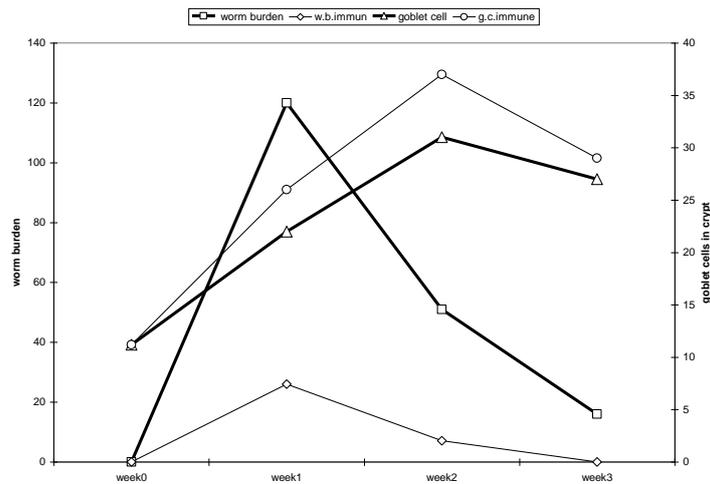


Figure (3): The change in the number of goblet cells in the crypts in relation to the worm burden, over 3 week period, in rats infected by *T.spiralis* with or without immunization by adult worm antigen. The values for the control were considered as week zero. The crypt goblet cell number is directly proportional to the worm burden. The highest value was obtained one week after the peak of the maximum worm burden.

The number of Mast cells

The number of mast cells per unit area in the lamina propria of the jejunum of infected rats was proportional to the increase in the worm period. The peak

however was obtained after two weeks of infection (table2, figure 4). In immunized rats the value of mast cell number was higher than in non-immunized. The increase at the peak value was 22.5%.

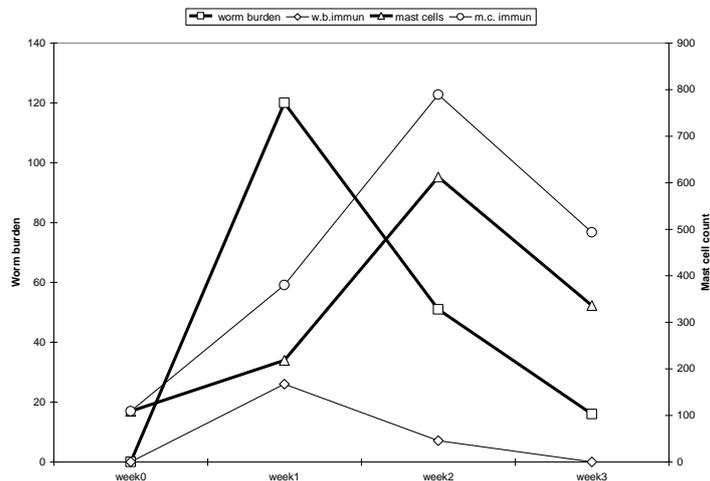


Figure (4): The change in the number of mast cells in intestinal mucosa in relation to the worm burden, over 3 week period, in rats infected by *T.spiralis* with or without immunization by adult worm antigen. The values for the control were considered as week zero. The mast cell number starts with a lag phase in the first week in non immunized rats. The number is higher without lag period in immunized rats. It is directly proportional to the worm burden. The highest value was obtained one week after the peak of the maximum worm burden.

The Histamine content

The histamine content was directly proportional to the mast cell number (figure 5).

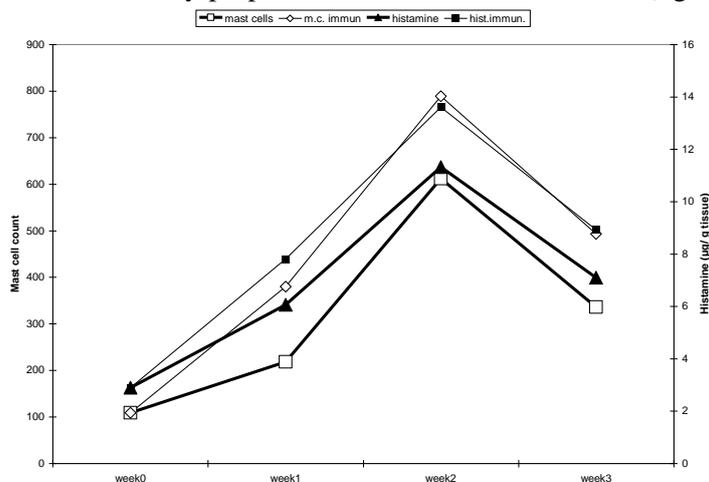


Figure (5): The change in the number of mast cells in intestinal mucosa in relation to histamine content, over 3 week period, in rats infected by *T.spiralis* with or without immunization by adult worm antigen. The values for the control were considered as week zero. The lag phase is less pronounced in the amount of histamine. The relationship between mast cell number and histamine content is clearly demonstrated.

As in mast cell number, immunization resulted in an increase in histamine (table 2). The increase, however, was slightly less than that of mast cell number (table 1)

suggesting an increase in cell proliferation not accompanied by similar increase in histamine secretion.

Table (1): Percent protection due to immunization of *T.spiralis* infected rats.

Parameter	worm burden	Larval emergence	Villus/Crypt ratio	Villus goblet cell count	Crypt goblet cell count	Mast cell count	Histamine content
% of non immunized on 2 nd wpi	67	38	78	13.3	16.22	22.5	16.8

Table (2): Mean numbers of goblet cells, mast cell /10 vcu of jejunal villi and Crypts and intestinal histamine in studied group

Groups	w.p.i	goblet cell/villus	Goblet cell/crypt	Mast cells 10vcu	Histamine content ug/ gm tissue
Control		19.5+1.6	11.2+1.1	109+23	2.89+1.23
non-immunized	1	36+03*	22+1.2*	218+36*	6.06+0.03*
	2	49.3+3.7*	612+17*	612+17*	11.32+3.1*
	3	22.3+1.8	336+28*	336+28*	7.1+1.6*
Immunized	1	42.6+0.1*	26+0.8*	380+18*	7.81+2.1*
	2	56.8+0.18*	37+1.8*	789+20*	13.61+2.4*
	3	24.1+0.03	29+0.2*	493+0.01*	8.92+0.06*

P<5.05-0.01 in GII and III compared by control using chi-square test

Discussion

Trichinosis can be fatal in the case of infection with high number of worms (Macleod, 1984). Trichinosis became of interest to Egyptian physicians after the recent discovery of *Trichinella spiralis* infection of lamb meat (Rosa *et al.*, 1992; Kim, 1993; and Theodoropoulos *et al.*, 2000).

The maximum number of worms were recorded in the intestinal mucosa one week post infection. This time coincides with the time needed for the development of sexually mature adults (Campbell *et al.*, 1999). Immunization with 2 successive subcutaneous injection of 100µg crude adult worm antigen 1 week apart followed by challenge infection by 300 infective *T. spiralis* larvae resulted in a significant reduction in worm burden compared to the infected non-immunized. The protection was 67%. In addition, 38% protection resulted from the effect on female worm fecundity with complete worm expulsion by the end of 3rd w.p.i. Similar results have been published by Ortega-Pierres *et al.* (1986) and Darwish *et al.* (1996).

The interpretation of the reduction in adult worm burden draws a clear conclusion that the immune status created by prior immunization enhances the overall protective immunity of the host against *T. spiralis* infection. This protection could be through several mechanisms including limitation of fecundity of female worms as indicated in the present work, or elimination of circulating newborn larvae but clearly not through rapid expulsion of adult worm. (Murrell, 1985). The antifecundity effect may be due to the gut immune response induced by the injected antigens (Murrell, 1985), inflammatory events in the intestinal tissues (Darwish *et al.*, 1996), and attack of the male organ, being cuticular in nature, by antibodies elicited by injected antigen will interfere with fertilization of the female with subsequent affection on fecundity (Ortega-Pierres *et al.*, 1986).

The morphological and morphometric measures of the intestinal mucosa of the studied groups showed that, in non-immunized rats, a significant decrease in

villus/crypt ratio is observed and reached its peak by the end of the 1st w.p.i. Gross inflammatory changes in the intestine could be involved in the shortening of the villi of infected group. Similar changes were also reported by Uber *et al.* (1980), Alizadeh & Wakelin (1982) and Sayed El-Ahl *et al.* (2001).

Expulsion of the adult worms of *T. spiralis* by the host seems to be an ambiguous multi factorial process but involvement of certain antibodies and cytokines is well recognized lately (Bell, 1998). In addition, there seems to be a possible role of epithelial cells in the protective immune response to *T. spiralis* manifested by observation of modified epithelial cell structure and function during a challenge infection. These changes included reduced wheat germ lectin binding to the epithelial brush border membrane in infected rats (Bell, 1998), reduced hexose transport (Hessel *et al.*, 1982), increased potential difference across epithelial tight junctions (Russell & Castro, 1985), and chloride ion secretion, which is linked to a change from fluid uptake to net fluid secretion by enterocytes (Bell, 1998). The functional changes in epithelial cells described in these studies have been linked to the immune system through immune serum transfer (Bell, 1998).

The number of goblet cells of infected rats progressively increased to reach a peak value by the end of the first week. Similar observations were reported by Friend *et al.* (1996) and Miller (1996). The increase in goblet cells mean number was accompanied by an increase in acid mucin. These quantitative and qualitative changes facilitated the expulsion of worms completely by the 3rd w.p.i in immunized group. Koninkx *et al.* (1988) attributed the increase of acid mucin to the increase in the number of sulfomucin-containing goblet cells. Khan *et al.* (1995) reported that intestinal mucus contributes to the immune-mediated prevention of establishment of parasites.

A significant increase in the mean number of mast cells was recorded over the three weeks of the experiment which indic-

ates the precise role played by these cells in worm expulsion. The effect of immunization was manifested in the form of enhancement of mastocytosis. Recently interpretations of mucosal mast cell involvement in worm expulsion were necessarily based upon quantitative data from histological preparations of intestinal tissue. Although such an approach allows correlations to be made between the kinetics of infection and changes in the number of mucosal mast cells present, yet it does not measure mucosal mast cell function directly. In the present study, mucosal mast cell activity was quantified by measuring histamine content in the intestinal tissues. In the gastrointestinal tract and at the cellular level, histamine is found largely in mast cells, mast cell like histaminocytes and in acid secreting glands in the stomach (Range & Dale, 1991). Release of cytokines has been proposed as a mechanism in immune response in trichinosis. Ridlinger *et al.* (1996) first characterized the cytokine profile in immunized mice including IL-2 and IL-3 which could induce mastocytosis and enhanced eosinophilia. Bell (1998) reported that IL-3 is the most important cytokine that can influence mast cell numbers. In conclusion, The histological and morphometric data support the parasitological data that immunization with worm antigen ameliorates the danger of trichinosis.

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الأستجابة المعوية للترايكينوزس فى الفأر الأبيض المحصن والغير المحصن

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لقد وجد ان هناك علاقة بين الغشاء المبطن للأمعاء الدقيقة و الإصابة بدودة الترايكينلاسيبراليز. فبينما يتأثر هذا الغشاء بتواجد الديدان و غزوها له اثناء و ضع يرقاتها – فأن الديدان تتأثر بمايفرزها هذا الغشاء من جزيئات خلوية (سايتوكاينز) و نشاط مناعى ولقد وجد ان التحصين بمحفزات للأجسام المضادة (الانتيجن) مستخلصة من عضلات اليرقات غير مجدى فى مقاومة هذه الدودة . غير انه من المتوقع ان يكون للتحصين بمستخلص كلى للدودة البالغة اكثر تأثيرا ضد الإصابة بهذه الدودة

بههدف هذا البحث الى دراسة العلاقة بين التركيب الهستولوجى و التغيرات فى مدى الاستجابة المناعية و الفياسات المورفولوجية لمخاطية الأمعاء (فى حالة الإصابة بالترايكينلا وحدها او الإصابة بعد التحصين بمستخلص الديدان البالغة) من ناحيه و بين التكاثر العددي والقدرة على انجاب اليرقات من ناحيه اخرى فى كل من الحيوانات المصابة معمليا بالديدان و تلك المحصنة بمستخلص الديدان البالغة قبل اصابتها .

حيث من المتوقع ان تؤدي مثل هذه الدراسة الى تقييم أفضل لتأثير التحصين بهذا المستخلص.

اسخدم فى هذا البحث 45 جرزا امهق بالغ قسمت الى ثلاث مجموعات : المجموعه الضابطه _ المجموعه المصابه معمليا بالترايكينلا سبيراليز . و المجموعه المحصنه قبل اصابتها بالديدان .

ولقد تم ذبح خمس فئران من كل مجموعة عند نهاية كل من الاسبوعين الاول والثانى و الثالث بعد الإصابة وقد تم تقييم تأثير التحصين اسبوعيا بقياس التواجد العددي للديدان البالغة والقدرة على انجاب اليرقات وعلاقتها بالتغيرات فى الشكل الهستولوجى والطول النسبى للخملات بالنسبة لغدد ليبركيين وعدد الخلايا الكاسية والخلايا المتخمة وكذلك كمية الهستامين فى النسيج المعوى.

وقد بينت النتائج أن التواجد العددي للديدان البالغة وصل الى قمة فى نهاية الاسبوع الأول بعد الإصابة حيث كانت نسبة الوقاية فى الفئران المحصنة 76% كما كانت الوقاية بقياس القدرة على انجاب اليرقات 38% بعد التحصين . ولقد أظهرت القياسات على مخاطية الأمعاء ان استجابة الأنسجة للأصابة قد وصلت قمتها فى نهاية الاسبوع الثانى – اى بعد اسبوع من قمة التواجد العددي للديدان البالغة .

واظهرت النتائج أن الوقاية بالتحصين فى الطول النسبى للخملات كان 78% بينما كان فى عدد الخلايا الكاسية فالخملات 13,3% وفى عدد ليبركين 16,22% وكانت القيمة بالنسبة للخلايا المنخمة 22,5% بينما كانت فى كمية الهستامين 16,8%

وتدل النتائج على أن التحصين بالمستخلص الكلى لديدان الترايكينلا سبيراليز البالغة يعطى وقاية لمخاطية أمعاء الحيوانات المصابة نتيجة للتأثير على تحور الديدان وانتاج اليرقات وقد يكون لافراز المخاط الحمضى من الخلايا الكاسية والهستامين من الخلايا المتخمة أثر فى هذه الوقاية .