

Acute trichinellosis increases susceptibility to *Giardia lamblia* infection in the mouse model

N. von ALLMEN¹, S. CHRISTEN¹, U. FORSTER², B. GOTTSTEIN¹, M. WELLE^{2*} and N. MÜLLER^{1*}

Institutes of Parasitology¹ and Veterinary Pathology², Vetsuisse Faculty, University of Berne, P.O. Box 8466, CH-3001 Berne, Switzerland

(Received 2 February 2006; revised 27 February 2006; accepted 28 February 2006; first published online 8 May 2006)

SUMMARY

The intestinal protozoan parasite *Giardia lamblia* causes diarrhoea in humans and animals. In the present study, we used the C57BL/6 inbred mouse model to assess the impact of a nematode (*Trichinella spiralis*) infection on the course of a *G. lamblia* (clone GS/M-83-H7) infection. Acute trichinellosis coincided with transient intestinal inflammation and generated an intestinal environment that strongly promoted growth of *G. lamblia* trophozoites although the local anti-*Giardia* immunoglobulin (Ig) A production was not affected. This increased *G. lamblia* infection intensity correlated with intestinal mast cell infiltration, mast cell degranulation, and total IgE production. Furthermore, a *G. lamblia* single-infection investigated in parallel also resulted in intestinal mast cell accumulation but severe infiltration was triggered in the absence of IgE. Recently, intestinal mast cells emerging during a *G. lamblia* infection were reported to be involved in those immunological mechanisms that control intestinal proliferation of the parasite in mice. This anti-giardial activity was assumed to be related to the capacity of mast cells to produce IL-6. However, this previous assumption was questioned by our present immunohistological findings indicating that murine intestinal mast cells, activated during a *G. lamblia* infection were IL-6-negative. In the present co-infection experiments, mast cells induced during acute trichinellosis were not able to control a concurrent *G. lamblia* infection. This observation makes it feasible that the *T. spiralis* infection created an immunological and physiological environment that superimposed the anti-giardial effect of mast cells and thus favoured intestinal growth of *G. lamblia* trophozoites in double-infected mice. Furthermore, our findings raise the possibility that intestinal inflammation e.g. as a consequence of a 'pre-existing' nematode infection is a factor which contributes to increased susceptibility of a host to a *G. lamblia* infection. The phenomenon of a 'pre-existing' nematode infection prior to a *G. lamblia* infection is a frequent constellation in endemic areas of giardiasis and may therefore have a direct impact on the epidemiological situation of the disease.

Key words: *Giardia lamblia*, giardiasis, *Trichinella spiralis*, trichinellosis, intestinal immune response, intestinal inflammation.

INTRODUCTION

Giardia lamblia (syn. *Giardia duodenalis*, *Giardia intestinalis*) is a common intestinal protozoan and causes diarrhoea in humans and animals. In many individuals, the infection remains asymptomatic, whereas some patients exhibit severe symptoms such as abdominal pain, nausea, as well as severe watery diarrhoea as a consequence of malabsorption (e.g. reviewed by Adam, 1991). In many cases, spontaneous resolution of the infection occurs after a few weeks but the disease may also develop to the chronic stage. The outcome of the infection is supposed to largely depend on the immunological status of

the infected individual but non-immunological factors are also involved in the interaction between the host and the parasite (e.g. reviewed by Faubert, 2000; Eckmann, 2003; Müller and von Allmen, 2005).

In giardiasis, reinfections are common because acquired immunity against *G. lamblia* is not complete either due to insufficient immune defences or antigenic variation of the parasite. Many studies in natural and experimental rodent hosts addressed the question whether antibodies and, more specifically, local secretory immunoglobulin (Ig) A antibodies play a role in control of the parasite infection (Eckmann, 2003). Previous findings in the experimental mouse/*G. lamblia* GS/M-83-H7 model indicated that B-cell-deficient animals were unable to clear giardial infections (Stäger and Müller, 1997; Langford *et al.* 2002). Conversely, by comparing the courses of infection in IL-6-deficient and wild-type mice, no obvious correlation between intestinal IgA production and the elimination of the parasite in the

* Corresponding authors: Institutes of Parasitology (N.M.) and Veterinary Pathology (M.W.), Länggass-Str. 122, CH-3012 Berne, Switzerland. Tel. +41 31 631 2474 (N.M.), +41 31 631 2416 (M.W.); Fax: +41 31 631 2477 (N.M.), +41 31 631 2635 (M.W.). E-mail: nmueller@ipa.unibe.ch (N.M.); monika.welle@itpa.unibe.ch (M.W.)

duodenum was observed (Bienz *et al.* 2003; Zhou *et al.* 2003). Data from an investigation based on the use of different transgenic mouse strains indicated that an as yet unknown T-cell-dependent mechanism is essential for controlling the acute phase of a *G. lamblia* infection (Singer and Nash, 2000).

Although the investigations listed above could not completely elucidate the cellular network involved in anti-giardial immune defence it became evident that antibody-independent immune effector mechanisms directly, or indirectly, interfere with maintenance and growth of the intestinal parasite population particularly during the acute phase of the infection (Singer and Nash, 2000; Langford *et al.* 2002; Li *et al.* 2004, 2006). Several investigations in the past provided evidence that mast cells are substantially involved in intestinal elimination of *Giardia* (Mitchell *et al.* 1982; Erlich *et al.* 1983; Li *et al.* 2004). Li *et al.* (2004) found that mast cells are activated during a *G. lamblia* infection and that mast cell-deficient, or -depleted, C57BL/6 mice, failed to control such an infection. In this report, mast cells were considered to be a potential source for IL-6. Since in the abovementioned (Li *et al.* 2004) and other studies (Bienz *et al.* 2003; Zhou *et al.* 2003) murine IL-6-deficiency was associated with a much increased susceptibility to a *G. lamblia* clone GS/M-83-H7 infection, mast cell-derived IL-6 was suggested to be important for the control of the respective infection.

As outlined above, the immunological parameters of a *G. lamblia* infection have mostly been determined in experimental hosts. However, only very few of these investigations took into account that the outcome of giardiasis may be influenced by other infections that had eventually occurred prior to the *G. lamblia* infection. Although not necessarily clinically relevant, such infections may alter the immunological and physiological intestinal environment and thus increase, or reduce, the susceptibility of a host to a subsequent *G. lamblia* infection. The existence of such a phenomenon was exemplified in a former investigation that assessed various parameters of a double infection of mice with the gut-dwelling nematode *Trichinella spiralis* and *Giardia muris* (Roberts-Thompson *et al.* 1976). As evidenced by multiple studies, *T. spiralis* infection is accompanied by Th2 cell-mediated eosinophilia and IgE production (Rosenberg *et al.* 1971; Matossian *et al.* 1977; Morakote *et al.* 1992; Finkelmann *et al.* 1997; Watanabe *et al.* 2005). During the early-infective, intestinal phase, *T. spiralis* triggers recruitment of inflammatory cells in the mucosa (Friend *et al.* 1996), and a subsequent worm expulsion is an immune-mediated process involving activation of Th2-cells (Faulkner *et al.* 1997; Finkelmann *et al.* 1997; Urban *et al.* 2000) and mast cells (Grencis *et al.* 1993; Kamiya *et al.* 1985; Donaldson *et al.* 1996). In the co-infection

experiment performed by Roberts-Thompson *et al.* (1976), *T. spiralis*-induced mucosal inflammation was associated with a strong reduction of the *G. muris* parasite load. This reduction in the giardial infection intensity turned out to be proportional to the mucosal inflammatory response that had emerged as a consequence of the *T. spiralis* infection.

Referring to these findings, we now performed a study to assess the impact of a murine *T. spiralis* infection on the course of a *G. lamblia* infection. For the infection experiments, we used the well-characterized *G. lamblia* clone GS/M-83-H7 (Aggarwal *et al.* 1989). As expected, 'pre-infection' of mice with *T. spiralis* larvae resulted in a transient mucosal infiltration of inflammatory cells (eosinophils and mast cells). However, in contrast to the *T. spiralis*/*G. muris* co-infection experiment described by Roberts-Thompson *et al.* (1976), intestinal inflammation during a *T. spiralis*/*G. lamblia* co-infection was accompanied by a transient massive increase of the *Giardia* parasite load. These results indicated that intestinal inflammatory reactions, induced by a *T. spiralis* infection, and more specifically *T. spiralis*-induced mucosal mast cell functions, did not contribute to the elimination of a *G. lamblia* GS/M-83-H7 infection.

MATERIALS AND METHODS

Animals

C57BL/6 mice were obtained from Charles River GmbH (Germany). The study was performed with 4 week-old female mice that were kept under specific pathogen-free (SPF) conditions according to Swiss regulations governing animal experimentation and rules for animal protection that restrict the use of experimental animals to a minimum.

Parasite and experimental infection

The origin, axenization and cloning of *G. lamblia* GS/M-83-H7 has been described by Aggarwal *et al.* (1989). *G. lamblia* trophozoites were cultivated in TYI-S-33 medium with antibiotics as previously described (Keister, 1983).

Experimental *G. lamblia* GS/M-83-H7 infections (6 animals per experimental time-point) were done with 10^6 trophozoites (suspended in 200 μ l of a 0.3 M NaHCO₃ solution) of *G. lamblia* GS/M-83-H7 using a blunt-ended needle for peroral inoculation. The course of the *G. lamblia* infection within mice was determined according to Gottstein *et al.* (1993) by quantitating the parasite burden through microscopical examination of adherent trophozoites from intestinal washes.

T. spiralis (strain ISS 384) was maintained through consecutive passages in outbred CD[®]-1(ICR)BR mice which were purchased from Charles

River GmbH (Germany). Infectious larvae (L1) were prepared by artificial digestion of muscle tissue from infected carcasses with 1% pepsin (Sigma-Aldrich, St Louis, MO) and 1% HCl and subsequent repeated washing in H₂O as described by Friend *et al.* (1996). The released larvae were microscopically counted and 300 larvae were resuspended in 50 µl of phosphate-buffered saline (PBS), pH 7.2, and were then applied for peroral infection of mice using a blunt-ended needle. Seven weeks after *T. spiralis* infection (corresponding to experimental time-point week 6 p.G.i.), infectivity of the larvae was confirmed by demonstrating that the means of the total carcass larval counts within the *T. spiralis*-infected and *T. spiralis*/*G. lamblia* double-infected animal groups was 6413 (range: 4360–7900; s.e.: ± 634) and 5046 (range: 4080–6800; s.e.: ± 464) larvae per animal. The efficiency of the *T. spiralis* infection was determined at a time-point when muscle larvae were expected to be fully developed (6–7 weeks after *T. spiralis* infection).

Determination of intestinal mast cells and eosinophils

Mice were sacrificed by CO₂ euthanasia. At necropsy, approximately 0.5 cm of the small intestine was fixed in formalin and embedded in paraffin wax. Serial sections of 4 µm thickness were cut from each tissue block. One section of each deparaffinized tissue sample was stained with the Luna's method (Prophet *et al.* 1994) to assess the number of eosinophilic granulocytes.

The mast cell numbers were determined with an enzyme-histochemical reaction for the detection of chymase activity with a commercially available detection kit (Sigma, Catalogue No. 91-C) using Naphthol-AS-D-Chloroacetate as substrate. The only modification to the kit protocol was the use of Fast Blue BB (Base) instead of Fast Red Violet LB (Base). Staining with Naphthol AS-D-Chloroacetate reveals mast cells as well as neutrophilic granulocytes and occasional macrophages. Discrimination especially between neutrophils and mast cells can be achieved by inspection of the shape and size of the nucleus. For this reason, only cells with a visible nucleus were counted in our experiments. Counting of the cells was performed by analysing the mucosa and submucosa of an entire section using a square eyepiece graticule. The number of mm² of the total area was assessed as well as the total cell number in this area. Cell numbers are expressed as cells/mm².

Immunohistological staining of IL-6-positive intestinal mast cells

For the detection of IL-6 produced in intestinal cells, immunohistochemical staining using a polyclonal

goat anti-rat IL-6 antibody (Santa Cruz Biotechnology, Santa Cruz, Ca, catalogue no. sc 1266) was performed. The specificity of the immunohistochemical staining was demonstrated with a blocking peptide (Santa Cruz Biotechnology, Santa Cruz, Ca, catalogue no. sc 1266P). In detail, slides were blocked (0.5% BSA-PBS/TBS, 10 min at room temperature) before the specimen was incubated with the primary antibody (diluted 1:35 in PBS) for 45 min at room temperature. This step was followed by incubation with a mouse anti-goat horseradish peroxidase conjugate (1:40 in PBS) for 20 min at room temperature. Endogenous peroxidase was quenched by incubating the section in H₂O₂ (1:10 in methanol) for 10 min at room temperature. Staining was completed by incubation with AEC Substrate-Chromogen for approximately 20 min at room temperature. Between each incubation step slides were washed twice for 5 min in PBS. Positive staining for IL-6 resulted in a red colour indicating the antigen localization of IL-6. Slides were counterstained with Ehrlich's haematoxylin and mounted in GVA mounting solution (Zymed Laboratories Inc. San Francisco CA, USA).

Determination of serum mast cell protease 1 and IgE levels

Serum concentrations of mouse mast cell protease 1 (MMCP-1) were determined as previously described by Li *et al.* (2004). Levels of serum IgE were determined by using the PharMingen IgE-capture ELISA (PharMingen, San Diego, CA; catalogue number 553413) according to the instructions of the manufacturer. IgE concentrations were extrapolated from a graph of standard OD_{405 nm}-values versus concentrations.

Determination of intestinal anti-*G. lamblia* IgA levels

For determination of intestinal anti-*Giardia* IgA concentrations, we applied the procedure described by Gottstein *et al.* (1993), which is suitable for the extraction of IgA from intestinal epithelium and lamina propria. Immunoreactivity of intestinal IgA antibodies was tested as previously described (Stäger *et al.* 1997), by using a soluble protein extract from *G. lamblia* clone GS/M-83-H7.

Statistical methods

The significance of the differences among the *G. lamblia* single-infected and the *T. spiralis*/*G. lamblia* double-infected animal groups was determined by the Student's *t*-test using the Microsoft Excel™ program. *P* values of <0.01 were considered statistically highly significant.

RESULTS

Intestinal Giardia lamblia proliferation in relation to inflammatory and local antibody reactions during primary Trichinella spiralis and secondary Giardia lamblia infection

To determine the impact of a *T. spiralis* infection on a subsequent *G. lamblia* GS/M-83-H7 infection, 4 week-old female mice either remained uninfected, or were perorally infected with 300 *T. spiralis* larvae (week -1, representing time-point at 1 week prior to *G. lamblia* infection). After 1 week (week 0, time-point at which animals were infected with *G. lamblia*), some of the previously uninfected, or *T. spiralis*-infected mice were perorally infected with 10^6 *G. lamblia* trophozoites. At different time-points corresponding to weeks -1, and 0, as well as weeks 1, 2, 3, and 6 post-*G. lamblia* infection (p.G.i.), 6 animals per experimental group (uninfected, *T. spiralis*-infected, *G. lamblia*-infected, and *T. spiralis*/*G. lamblia*-double-infected animals) were sacrificed by CO₂ euthanasia.

The efficiency of the *T. spiralis* infection was determined by assessing mean total carcass larval counts within the *T. spiralis*-infected and *T. spiralis*/*G. lamblia* double-infected animal groups at a time-point where muscle larvae were expected to be fully mature (7 weeks after *T. spiralis* infection). Mean larval counts did not significantly differ within the two experimental animal groups ($P=0.12$) and were between 6413 (range: 4368–7925; s.e.: ± 634) (*T. spiralis*-infected) and 5046 (range: 4080–6842; s.e.: ± 464) (*T. spiralis*/*G. lamblia* double-infected) per animal, respectively.

Regarding the course of the *G. lamblia* infection in *T. spiralis*-pre-infected or non-pre-infected animals, the following results were obtained: animals exclusively infected with *G. lamblia*, exhibited a low-intensity infection and trophozoites were only detectable at weeks 1 (mean: 4.6×10^4 parasites per cm duodenum) and 2 (mean: 0.2×10^4 parasites per cm duodenum) p.G.i. (Fig. 1A). Compared to these animals, *T. spiralis*-pre-infected animals exhibited an approximately 5-fold and 150-fold higher duodenal trophozoite burden at weeks 1 and 2 p.G.i., respectively. Furthermore, in double-infected animals residual trophozoites were still visible at weeks 3 (mean: 6.6×10^4 parasites per cm duodenum) and 6 (0.2×10^4 parasites per cm duodenum) p.G.i. Accordingly, pre-infection of mice with *T. spiralis* transiently promoted the intestinal *G. lamblia* trophozoite proliferation and thus up-shifted both intensity and duration of the *G. lamblia* infection.

In addition, we analysed various parameters linked to the intestinal inflammatory status of the different experimental animal groups in relation to the course of the *G. lamblia* infection in single- and double-infected mice. In particular, we found that the

T. spiralis infection resulted in a transient accumulation of eosinophils in the intestinal mucosa, which already reached its maximum around the time-point where the *G. lamblia* infection was initiated (week 0 p.G.i.) (Fig. 1B). Conversely, the *G. lamblia* infection alone did not lead to a significant eosinophil infiltration.

In *T. spiralis*-infected mice, mucosal mast cells started to accumulate around week 0 p.G.i. and reached a maximal density around week 3 p.G.i. (Fig. 1C). At week 6 p.G.i., only relatively small numbers of mast cells resided in the intestinal mucosa of these animals. In *G. lamblia*-single-infected mice, mucosal mast cell numbers were somewhat lower and peak levels were observed at week 2 p.G.i. before respective cell numbers dropped to basal levels during week 3 p.G.i. Interestingly, *T. spiralis*/*G. lamblia* double-infected mice exhibited mucosal mast cell numbers that at weeks 1 and 2 p.G.i. were significantly higher than those observed in *T. spiralis* or *G. lamblia* single-infected animals. Accordingly, consecutive infection of mice with *T. spiralis* and *G. lamblia* apparently had a synergistic effect on mast cell infiltration into the mucosa.

As assessed by Li *et al.* (2004), the serum MMCP-1 concentration is a suitable marker to monitor the degranulation status of intestinal mast cells activated during a *G. lamblia* infection. In the present study, infection of animals with *T. spiralis* resulted in an immediate and strong induction of serum MMCP-1 already detectable at week 0 p.G.i. (Fig. 1D). In both, *T. spiralis* single-infected and *T. spiralis*/*G. lamblia* double-infected mice, the serum MMCP-1 levels remained relatively high until week 3 p.G.i., and only slightly declined between weeks 3 and 6 p.G.i. Compared to these two experimental animal groups, *G. lamblia* single-infected animals only produced approximately 50% of serum MMCP-1 between weeks 1 and 2 p.G.i., and MMCP-1 production essentially did not extend beyond week 3 p.G.i.

As previously described, murine trichinellosis is associated with IgE production that is involved in mast cell homeostasis, and that partially triggers mast cell degranulation including the release of mediators such as MMCP-1 (Gurish *et al.* 2004). An assay monitoring serum total IgE levels revealed that serum from *T. spiralis* single-infected and *T. spiralis*/*G. lamblia* double-infected mice, but not serum from *G. lamblia*-infected and non-infected mice, contained detectable amounts of IgE (Fig. 1E). In the 2 positive groups, IgE production started around week 0 p.G.i. and extended the final time-point (week 6 p.G.i.) of the infection experiment. Interestingly, double-infected animals exhibited a marked rise in total IgE levels between weeks 0 and 1 p.G.i. whereas in *T. spiralis*-single-infected animals high-level serum IgE production started between weeks 1 and 2 p.G.i.

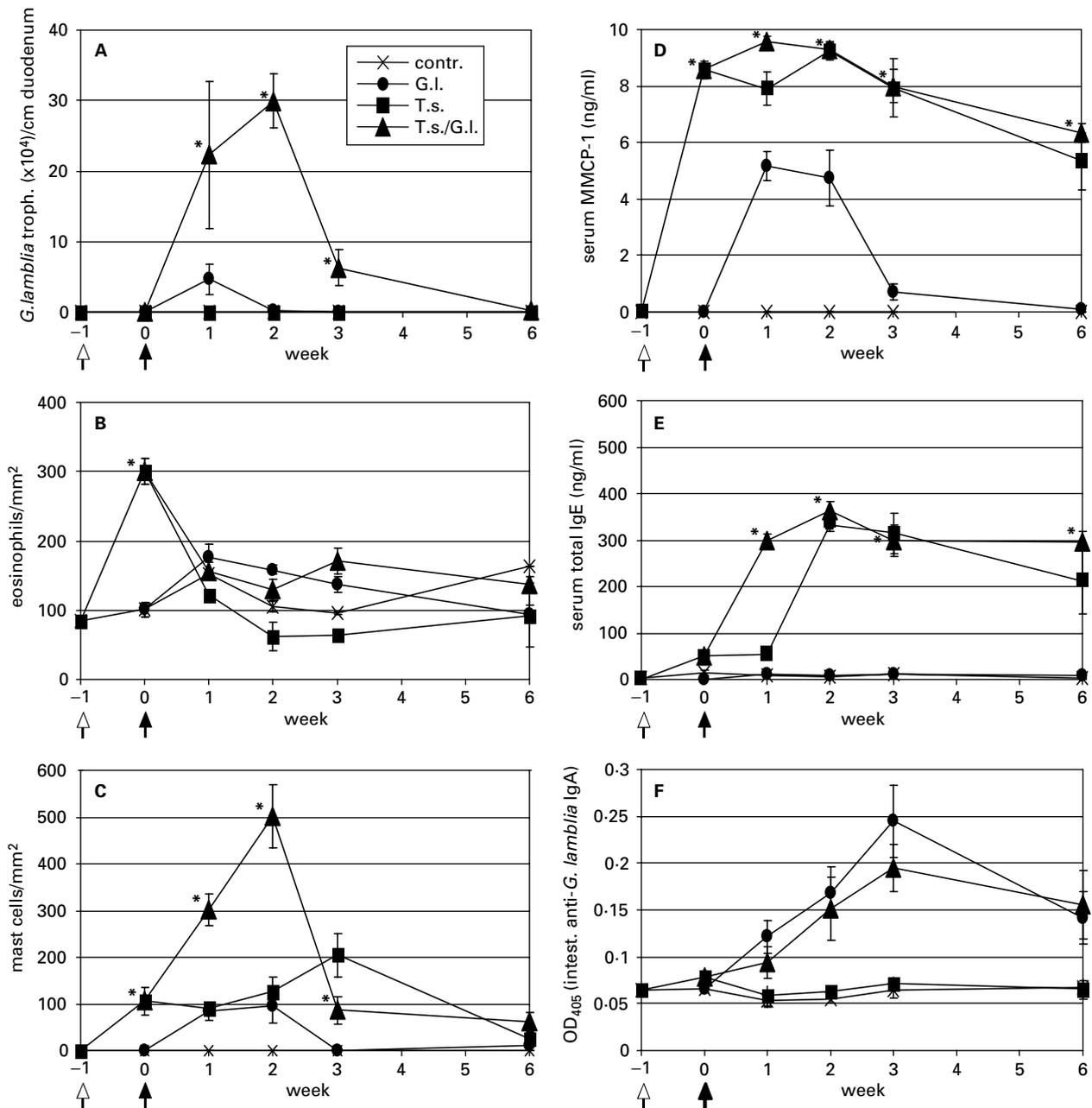


Fig. 1. Determination of intestinal *Giardia lamblia* trophozoite (A), intestinal eosinophil (B), and intestinal mast cell numbers (C), and serum MMCP-1 (D), serum total IgE (E), and intestinal anti-*G. lamblia* IgA levels in uninfected control (contr.), *G. lamblia* (*G.l.*), *T. spiralis* (*T.s.*), and *T. spiralis/G. lamblia* (*T.s./G.l.*) infected mice. Samples were taken at week -1 (time-point of *T. spiralis* infection as indicated by an arrow with an open head), week 0 (time-point of *G. lamblia* infection as indicated by an arrow with a closed head), and at weeks 1, 2, 3, and 6 post *G. lamblia* infection. Values are given as means (\pm s.e.) and highly significant differences between the values representing *G. lamblia*- and *G. lamblia/T. spiralis*-infected animal groups were assessed by using the Student's *t*-test ($*P < 0.01$).

Since in the current literature (e.g. reviewed by Eckmann, 2003) intestinal IgA is discussed as a possible immunological effector involved in the control of giardiasis, we decided to examine the different experimental animal groups in view of their intestinal anti-*G. lamblia* IgA-production. For this purpose, intestinal IgA was extracted from intestinal tissue samples and IgA-antibody reactivities to

soluble *G. lamblia* GS/M-83-H7 trophozoite antigen were determined by ELISA as described in the Materials and Methods section (Fig. 1F). In this analysis, both *G. lamblia* single- and *T. spiralis/G. lamblia* double-infected animals elicited a significant intestinal IgA response to *G. lamblia*. Respective antibody reactions were already detectable between 1 and 2 weeks p.G.i.

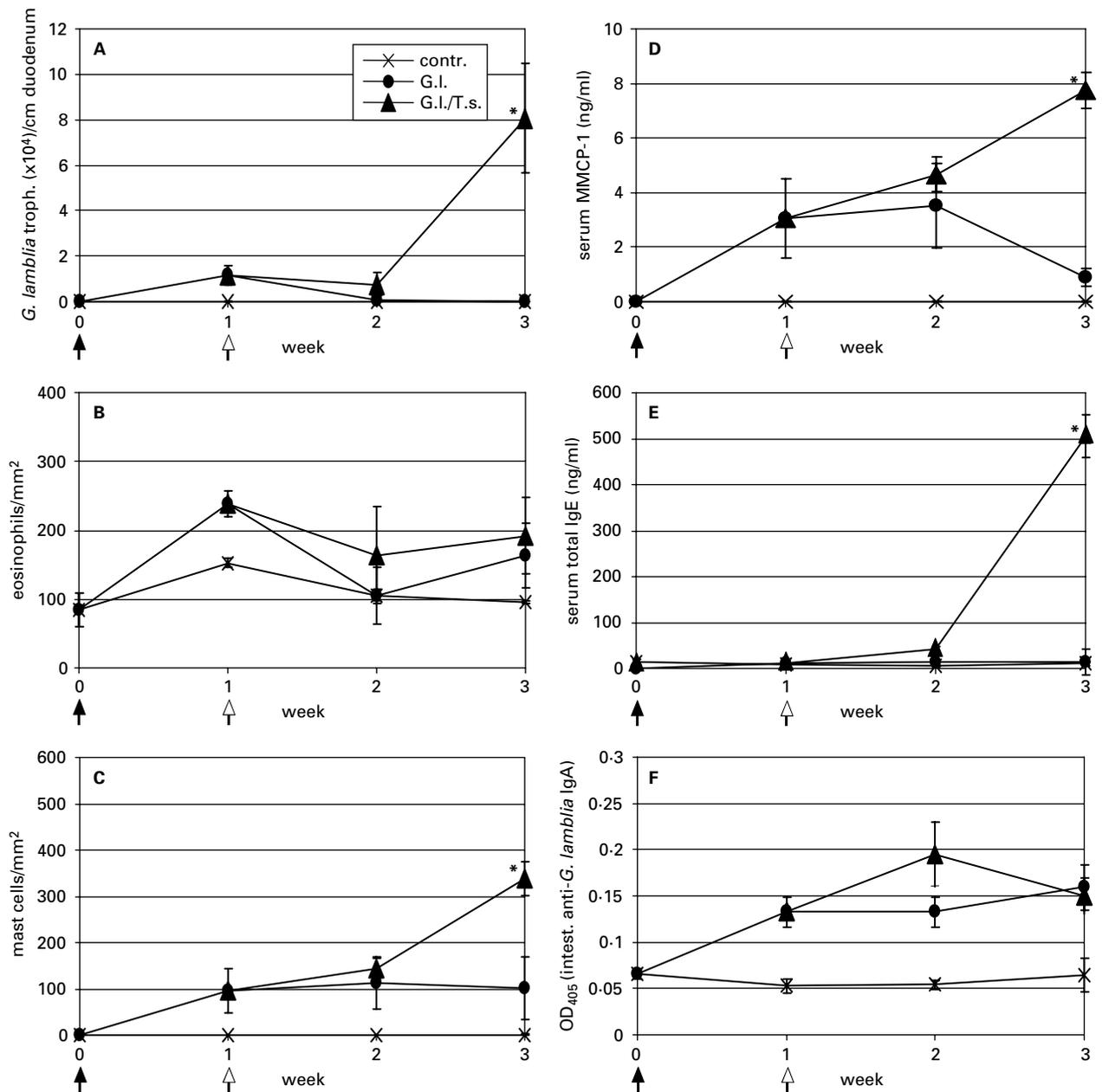


Fig. 2. Determination of intestinal *Giardia lamblia* trophozoite (A), intestinal eosinophil (B), and intestinal mast cell numbers (C), and serum MMCP-1 (D), serum total IgE (E), and intestinal anti-*G. lamblia* IgA levels in uninfected control (contr.), *G. lamblia* – (G.I.), and *G. lamblia*/*T. spiralis* – (G.I./T.s.) infected mice. Samples were taken at week 0 (time-point of *G. lamblia* infection as indicated by an arrow with a closed head), week 1 (time-point of *T. spiralis* infection as indicated by an arrow with an open head), and at weeks 2 and 3 post-*G. lamblia* infection. Values are given as means (\pm s.e.) and highly significant differences between the values representing *G. lamblia*- and *G. lamblia*/*T. spiralis*-infected animal groups were assessed by using the Student's *t*-test (* $P < 0.01$).

Intestinal Giardia lamblia proliferation in relation to inflammatory and local antibody reactions during primary Giardia lamblia and secondary Trichinella spiralis infection

To determine the impact of a *T. spiralis* infection on the course of a previous *G. lamblia* GS/M-83-H7 infection, 5-week-old female mice were perorally infected with 10^6 *G. lamblia* trophozoites (week 0). Some of these animals were sacrificed at time-points corresponding to weeks 1 (time-point at which

T. spiralis infection had been carried out) and 3 p.G.i. As shown in Fig. 2A, during the acute-phase of *G. lamblia* infection around week 1 p.G.i., exposure of the animals to a subsequent *T. spiralis* infection resulted in (re-)activation of the intestinal *G. lamblia* trophozoite growth within a following 2-week period. However, when the secondary *T. spiralis* infection was performed at later stages, no relapsing of an eventual residual *G. lamblia* population was observed (data not shown). The efficiency of the *T. spiralis* infection was determined by assessing mean total

carcass larval counts within the *T. spiralis*-infected and *T. spiralis/G. lamblia* double-infected animal groups at a time-point where muscle larvae were expected to be fully mature (6 weeks after *T. spiralis* infection, not shown). Mean larval counts did not significantly differ within the two experimental animal groups ($P=0.15$) and were between 6976 (range: 4865–7810; S.E.: ± 462) (*T. spiralis*-infected) and 5703 (range: 4025–7980; S.E.: ± 662) (*T. spiralis/G. lamblia* double-infected) per animal, respectively.

Comparative analyses of intestinal inflammation and local antibody production during *T. spiralis*-induced re-activation of a *G. lamblia* GS/M-83-H7 infection provided the following findings: while no massive intestinal accumulation of eosinophils was detected in any of the experimental animal groups (Fig. 2B), *G. lamblia* single- and *G. lamblia/T. spiralis* double-infected mice exhibited a significant increase of intestinal mast cells (Fig. 2C). As shown in Fig. 2D, this increase coincided with a mast cell degranulation pattern. Thus, at week 3 p.G.i., *G. lamblia/T. spiralis* double-infected animals contained approximately 9-fold higher MMCP-1 serum levels than *G. lamblia*-single infected animals. Furthermore, elevated serum total IgE production was restricted to double-infected animals and was only observed at week 3 of the co-infection experiment (Fig. 2E). Conversely, anti-*G. lamblia* IgA was detected in both *G. lamblia*-single and *G. lamblia/T. spiralis*-double-infected mice (Fig. 2F). In both animal groups, antibody concentrations reached similar levels and started around week 1 p.G.i.

Characterization of IL-6-producing intestinal cells in *Trichinella spiralis*/*Giardia lamblia* single- and double-infected mice

Intestinal mast cell-derived IL-6 may be important for control of a *G. lamblia* clone GS/M-83-H7 infection in mice (Li *et al.* 2004). In order to find out if a previous *T. spiralis* infection increases murine susceptibility to infection with *G. lamblia* clone GS/M-83-H7 and if this is associated with a reduced IL-6-positivity of intestinal mast cells, a combined histological/immunohistological investigation was carried out on formalin-fixed, paraffin-embedded intestinal tissue, representing the different experimental animal groups from week 2 p.G.i. In these histological sections, mast cells were visualized by chymase staining. In corresponding sections, IL-6-producing cells were detected with an IL-6-specific antibody and the specificity of the immunostaining was confirmed by demonstrating neutralization of the antibody reactivity with a blocking peptide (not shown).

Typical features from the different (immuno-) histological stainings as outlined above are shown in Fig. 3. Interestingly, the vast majority of intestinal mast cells in *G. lamblia* single-infected mice (Fig. 3A,

panel a) were located in the crypt area whereas mast cells in *T. spiralis* single-infected (Fig. 3B, panel a) and *T. spiralis/G. lamblia* double-infected (Fig. 3C, panel a) animals were located in the crypt area as well as in the villi. In uninfected control animals (Fig. 3D, panel a), no intestinal mast cells were detected. In intestinal tissue from *G. lamblia*-single-infected (Fig. 3A, panel b), *T. spiralis*-single-infected (Fig. 3B, panel b), *T. spiralis/G. lamblia*-double-infected (Fig. 3C, panel b), and uninfected (Fig. 3D, panel b) mice no anti-IL-6 antibody staining patterns were observed that may have reflected the distribution of intestinal mast cell infiltrates. In contrast, epithelial cells exhibited a specific and consistent reactivity with the anti-IL-6-antibodies, and in all experimental animal groups epithelial immunostaining was visible at about the same intensity. Conclusively, these results indicated that neither in single- and double-infected mice, nor in uninfected control mice, intestinal mast cells (and other intestinal cellular infiltrates) synthesized detectable amounts of IL-6. Conversely, intestinal epithelial cells seemed to produce IL-6 irrespective of the infection status of the animals investigated.

DISCUSSION

As recently reported, *G. lamblia* (clone GS/M-83-H7) infections in mice were associated with intestinal mast cell infiltration and this process seemed to be involved in resolution of infection (Li *et al.* 2004). The aim of the present study was to find out if intestinal mast cells – stimulated during a concurrent *T. spiralis* infection – are also able to modulate the course of such a *G. lamblia* infection. Our investigation was performed in analogy to a former *T. spiralis/G. muris* co-infection experiment that demonstrated a suppression of *G. muris* trophozoite proliferation as a consequence of a preceding *T. spiralis* infection (Roberts-Thompson *et al.* 1976). However, in contrast to the findings from this former study, our results revealed that a *T. spiralis* infection did not suppress, but rather stimulated, intestinal growth of a respective *G. lamblia* trophozoite population. The divergent outcome of these two analogous co-infection experiments may reflect differences in either the (immuno-)biology of the *G. lamblia* and the *G. muris* species or the genetic background and/or the age of the infected mice. However, the particular reasons for these differential giardial growth patterns under conditions of a concomitant *T. spiralis* infection still remain to be elucidated.

In our study, C57BL/6 mice pre-infected with *T. spiralis* demonstrated a transiently increased susceptibility to a *G. lamblia* (clone GS/M-83-H7) infection. Furthermore, the intensity of a primary *G. lamblia* infection was enhanced by secondary infection of animals with *T. spiralis*. In concordance

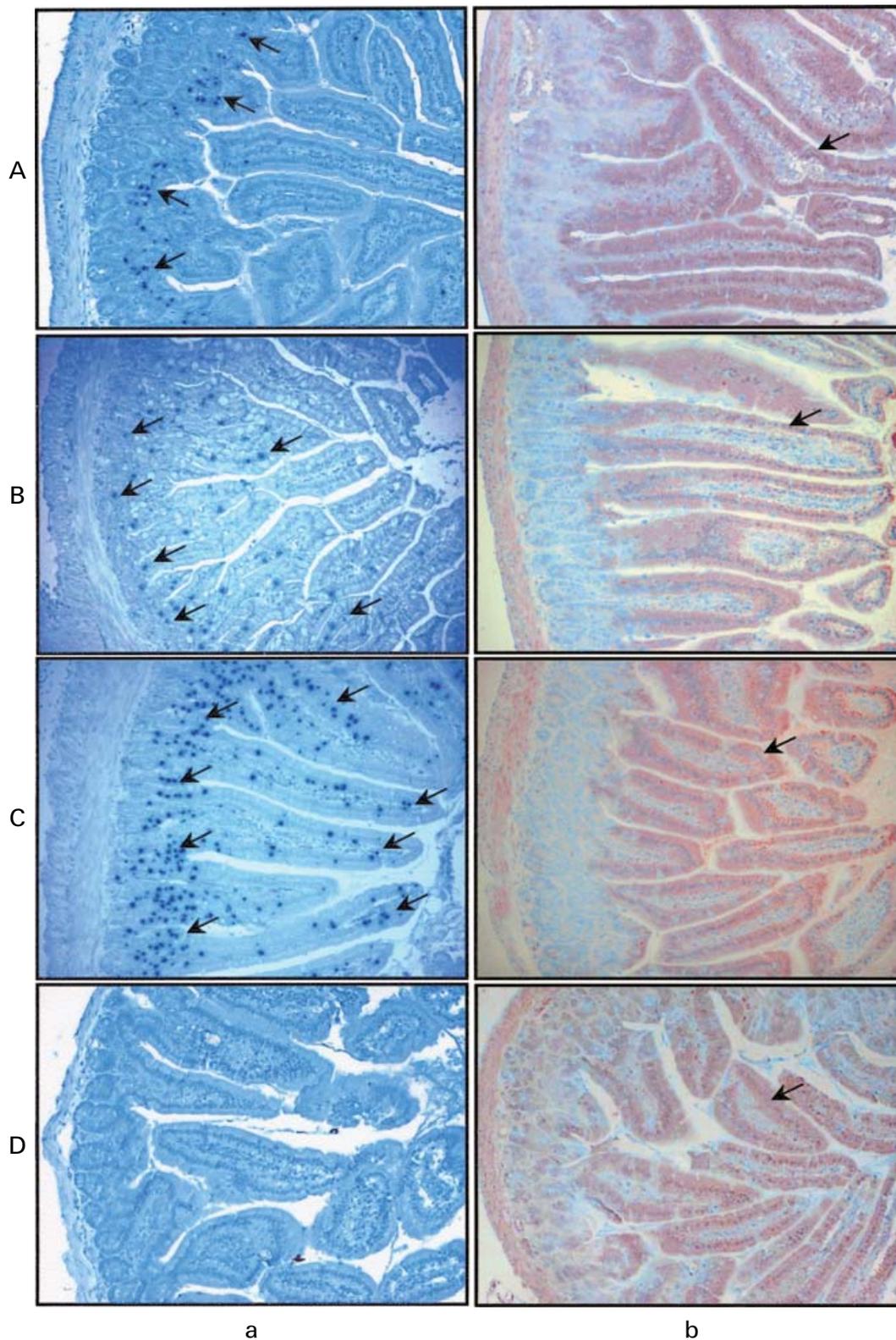


Fig. 3. Detection of intestinal mast cells and IL-6-producing cells in mice at week 2 post-*Giardia lamblia* infection. Enzyme-histochemical staining for mast cell chymase demonstrated different numbers of mast cells in *G. lamblia* – (A, panel a), *T. spiralis* – (B, panel a), and *T. spiralis/G. lamblia* – (C, panel a) infected mice but apparently no mast cells in the controls (D, panel a). Tissue areas with mast cell accumulations are indicated by arrows. Immunohistochemical staining using an anti-murine IL-6 antibody revealed a mild to moderate IL-6 expression in the epithelial cells of infected (A, B, and C, panel b) and control (D, panel b) mice. No IL-6 expression was detected in the areas of mast cell accumulation. Areas representing epithelial cell layers are indicated by arrows (magnification $\times 200$).

with previous data (Gurish *et al.* 2004), the present co-infection experiment in mice revealed that early phase *T. spiralis* infection coincided with transient intestinal inflammation and was particularly associated with a massive eosinophil and mast cell infiltration into the mucosa, mast cell degranulation, and IgE production. Intestinal eosinophil infiltration was visible only during a short period (approximately 1 week) during the initial stage of the *T. spiralis* infection and thus most likely did not have a substantial influence on the course of the secondary *G. lamblia* infection. Conversely, in our co-infection experiments, intestinal accumulation of mast cells was observed during a much longer period (approximately 3 weeks) and this phenomenon strikingly coincided with a transient increase of the intestinal *G. lamblia* trophozoite burden. Thus, it became evident that mast cells emerging within the intestinal inflammatory environment during an acute *T. spiralis* infection were not able to control a concurrent *G. lamblia* infection. Rather, our findings suggested that these mast cells might have contributed to the enhanced growth of the intestinal *G. lamblia* population. However, this hypothesis is challenged by data from a recent study demonstrating that mast cells induced by *G. lamblia* display an inhibitory effect on giardial proliferation in mice (Li *et al.* 2004). In combination, the previous and present results outlined above may indicate that murine mast cells induced by either *T. spiralis* or *G. lamblia* were involved in functionally distinct immunological processes that either promoted, or suppressed, intestinal growth of *G. lamblia* trophozoites. Since both of these putatively distinct mast cell populations turned out to release MMCP-1, we concluded that the differential effect on giardial growth was probably not related to differential degranulation characteristics of these particular cells.

One possible mechanism for mast cell activation is dependent on IgE and Fc ϵ -receptor I although other factors such as IL-10 are involved in the degranulation process as well (Ghildyal *et al.* 1992). Activation of mast cells by IgE is supposed to elicit the release of an array of cytokines and chemokines that are involved in regulation of the acute inflammatory response (Gurish *et al.* 2004). Interestingly, in our investigation the *G. lamblia* infection was found to trigger MMCP-1 release in the absence of IgE, whereas in *T. spiralis*-infected mice this process occurred in the presence of IgE. This finding certainly needs further attention and future investigations e.g. based on the use of an IgE-deficient mouse model will have to explore whether production of IgE has a negative effect on anti-giardial immunity and increases susceptibility of the animals to the parasite infection. Respective results will be particularly interesting because a correlation between increased IgE production and susceptibility to symptomatic giardiasis has already been observed in an allergic

versus non-allergic group of Venezuelan children (Di Prisco *et al.* 1993).

As reported formerly, epithelial cells (McGee *et al.* 1993) but also mast cells (e.g. reviewed by Galli *et al.* 2005) have the capacity to express IL-6. Furthermore, previous experimental *G. lamblia* infections in IL-6-deficient mice clearly evidenced a central role of IL-6 in anti-giardial immunity (Bienz *et al.* 2003; Zhou *et al.* 2003). These observations are in agreement with recent data from Li *et al.* (2004) suggesting that IL-6-producing mast cells may be key players within the intestinal immunological network that mediates immunity against a *G. lamblia* infection in mice. However, this assumption is challenged by our present immunohistological data indicating that murine mast cells, induced by a *G. lamblia* infection, seemed not to produce IL-6 in a quantity above the level of IL-6 production of intestinal epithelial cells from uninfected, or *G. lamblia*-infected, mice. Our conclusion was also confirmed by results from a quantitative reverse transcription (RT-)-PCR approach, which did not demonstrate increased intestinal IL-6 expression upon infection of mice with *G. lamblia* (data not shown). The conflicting results listed above make it evident that further extensive experimental work will be needed to elucidate the IL-6- and mast cell-dependent processes that contribute to murine anti-giardial immunity.

Intestinal mucosal inflammation has already previously been evaluated regarding its importance in resistance to murine giardiasis (e.g. reviewed by Müller and von Allmen, 2005). For example, a study on *G. muris* infections in a resistant versus susceptible mouse model did not evidence a correlation between resistance against infection and mucosal inflammation (Venkatesan *et al.* 1997). Conversely, Li *et al.* (2004) demonstrated that accumulation of inflammatory mast cells contributed to the elimination of a *G. lamblia* infection in the experimental murine host. Finally, our present data indicated that a *T. spiralis* infection, and perhaps particularly the intestinal inflammation induced by this infection, favours establishment and maintenance of a *G. lamblia* population in mice. It is obvious that in natural hosts, a *G. lamblia* infection often occurs in a situation where the intestinal tissue is inflamed e.g. as a consequence of a 'pre-existing' nematode infection. Nematode infections are evidently widespread in most endemic areas of giardiasis (Gendrel *et al.* 2003; Owen, 2005) and the phenomenon of a 'pre-existing' nematode infection prior to a *G. lamblia* infection may therefore have a direct influence on the epidemiological relevance of the disease.

As for example demonstrated for nematode infections, the inflammatory process involved in intestinal (immuno-)pathogenesis can change both the immunological and physiological conditions inside the intestinal environment (Garside *et al.*

2000; Lawrence, 2003; Khan and Collins, 2004; Maizels *et al.* 2004). Inflammation-associated modulation of the Th1/Th2 balance, alterations in intestinal nitric oxide, mucin and defensin production, and alterations in the composition of the intestinal flora may influence proliferation and persistence of *G. lamblia* trophozoites in the gut (e.g. reviewed by Müller and von Allmen, 2005). Accordingly, approaches investigating giardial growth in relation to an eventual intestinal inflammatory reaction will provide novel information on the immunological and physiological functions that promote either resistance or susceptibility to a *G. lamblia* infection.

We acknowledge U. Brönnimann, H. Sager (Institute of Parasitology), M. Bozzo, E. Garchi, and E. Rohner for technical support, and T. E. Nash (NIH, Bethesda, Maryland, USA) for his gift of *G. lamblia* clone GS/M-83-H7. This work was financed through a grant obtained from the Swiss National Science Foundation (No. 31-066795.01).

REFERENCES

- Adam, R. D.** (1991). The biology of *Giardia* spp. *Microbiological Reviews* **55**, 706–732.
- Aggarwal, A., Merritt, J. W. and Nash, T. E.** (1989). Cysteine-rich variant surface proteins of *Giardia lamblia*. *Molecular and Biochemical Parasitology* **32**, 39–48.
- Bienz, M., Dai, W. J., Welle, M., Gottstein, B. and Müller, N.** (2003). Interleukin-6-deficient mice are highly susceptible to *Giardia lamblia* infections but exhibit normal intestinal IgA responses against the parasite. *Infection and Immunity* **71**, 1569–1573.
- Di Prisco, M. C., Hagel, I., Lynch, N. R., Barrios, R. M., Alvarez, N. and Lopez, R.** (1993). Possible relationship between allergic disease and infection by *Giardia lamblia*. *Annals of Allergy* **70**, 210–213.
- Donaldson, L. E., Schmitt, E., Huntley, J. F., Newlands, G. F. and Grecis, R. K.** (1996). A critical role for stem cell factor and c-kit in host protective immunity to an intestinal helminth. *International Immunology* **8**, 559–567.
- Eckmann, L.** (2003). Mucosal defences against *Giardia*. *Parasite Immunology* **25**, 259–270.
- Erlich, J. H., Anders, R. F., Roberts-Thomson, I. C., Schrader, J. W. and Mitchell, G. F.** (1983). An examination of differences in serum antibody specificities and hypersensitivity reactions as contributing factors to chronic infection with the intestinal protozoan parasite, *Giardia muris*, in mice. *Australian Journal of Experimental Biology and Medical Sciences* **61**, 599–615.
- Faubert, G. M.** (2000). Immune response to *Giardia duodenalis*. *Clinical Microbiology Reviews* **13**, 35–54.
- Faulkner, H., Humphreys, N., Renauld, J. C., Van Snick, J. and Grecis, R.** (1997). Interleukin-9 is involved in host protective immunity to intestinal nematode infection. *European Journal of Immunology* **27**, 2536–2540.
- Finkelman, F. D., Shea-Donohue, T., Goldhill, J., Sullivan, C. A., Morris, S. C., Madden, K. B., Gause, W. C. and Urban, J. F. Jr.** (1997). Cytokine regulation of host defense against parasitic gastrointestinal nematodes: lessons from studies with rodent models. *Annual Review of Immunology* **15**, 505–533.
- Friend, D. S., Ghildyal, N., Austen, K. F., Gurish, M. F., Matsumoto, R. and Stevens, R. L.** (1996). Mast cells that reside at different locations in the jejunum of mice infected with *Trichinella spiralis* exhibit sequential changes in their granule ultrastructure and chymase phenotype. *Journal of Cell Biology* **135**, 279–290.
- Friend, D. S., Gurish, M. F., Austen, K. F., Hunt, J. and Stevens, R. L.** (2000). Senescent jejunal mast cells and eosinophils in the mouse preferentially translocate to the spleen and draining lymph node, respectively, during the recovery phase of helminth infection. *Journal of Immunology* **165**, 344–352.
- Galli, S. J., Nakae, S. and Tsai, M.** (2005). Mast cells in the development of adaptive immune responses. *Nature Immunology* **6**, 135–142.
- Garside, P., Kennedy, M. W., Wakelin, D. and Lawrence, C. E.** (2000). Immunopathology of intestinal helminth infection. *Parasite Immunology* **22**, 605–612.
- Gendrel, D., Treluyer, J. M. and Richard-Lenoble, D.** (2003). Parasitic diarrhea in normal and malnourished children. *Fundamental and Clinical Pharmacology* **17**, 189–197.
- Ghildyal, N., McNeil, H. P., Stechschulte, S., Austen, K. F., Silberstein, D., Gurish, M. F., Somerville, L. L. and Stevens, R. L.** (1992). IL-10 induces transcription of the gene for mouse mast cell protease-1, a serine protease preferentially expressed in mucosal mast cells of *Trichinella spiralis*-infected mice. *Journal of Immunology* **149**, 2123–2129.
- Gottstein, B., Deplazes, P. and Tanner, I.** (1993). In vitro synthesized immunoglobulin A from nu/+ and reconstituted nu/nu mice against a dominant surface antigen of *Giardia lamblia*. *Parasitology Research* **79**, 644–648.
- Grecis, R. K., Else, K. J., Huntley, J. F. and Nishikawa, S. I.** (1993). The in vivo role of stem cell factor (c-kit ligand) on mastocytosis and host protective immunity to the intestinal nematode *Trichinella spiralis* in mice. *Parasite Immunology* **15**, 55–59.
- Gurish, M. F., Bryce, P. J., Tao, H., Kisselgof, A. B., Thornton, E. M., Miller, H. R., Friend, D. S. and Oettgen, H. C.** (2004). IgE enhances parasite clearance and regulates mast cell responses in mice infected with *Trichinella spiralis*. *Journal of Immunology* **172**, 1139–1145.
- Kamiya, M., Oku, Y., Itayama, H. and Ohbayashi, M.** (1985). Prolonged expulsion of adult *Trichinella spiralis* and eosinophil infiltration in mast cell-deficient W/W^v mice. *Journal of Helminthology* **59**, 233–239.
- Keister, D. B.** (1983). Axenic culture of *Giardia lamblia* in TYI-S-33 medium supplemented with bile. *Transaction of the Royal Society of Tropical Medicine and Hygiene* **77**, 487–488.
- Khan, W. I. and Collins, S. M.** (2004). Immune-mediated alteration in gut physiology and its role in host

- defence in nematode infection. *Parasite Immunology* **26**, 319–326.
- Langford, T. D., Housley, M. P., Boes, M., Chen, J., Kagnoff, M. F., Gillin, F. D. and Eckmann, L.** (2002). Central importance of immunoglobulin A in host defense against *Giardia* spp. *Infection and Immunity* **70**, 11–18.
- Lawrence, C. E.** (2003). Is there a common mechanism of gastrointestinal nematode expulsion? *Parasite Immunology* **25**, 271–281.
- Li, E., Zhou, P., Petrin, Z. and Singer, S.** (2004). Mast cell-dependent control of *Giardia lamblia* infections in mice. *Infection and Immunity* **72**, 6642–6649.
- Li, E., Zhou, P. and Singer, S.** (2006). Neuronal nitric oxide synthetase is necessary for elimination of *Giardia lamblia* infections in mice. *Journal of Immunology* **176**, 516–521.
- Maizels, R. M., Balic, A., Gomez-Excobar, N., Nair, M., Taylor, M. D. and Allen, J. E.** (2004). Helminth parasites-masters of regulation. *Immunological Reviews* **201**, 89–116.
- Matossian, R. M., Salti, I. and Stephan, E.** (1977). Variation in serum immunoglobulin levels in acute trichinosis. *Journal of Helminthology* **51**, 1–4.
- McGee, D. W., Elson, C. O. and McGee, J. R.** (1993). Enhancing effect of cholera toxin on interleukin-6 secretion by IEC-6 intestinal epithelial cells: mode of action and augmentation effect of inflammatory cytokines. *Infection and Immunity* **61**, 970–978.
- Mitchell, G. F., Anders, R. F., Brown, G. V., Handman, E., Roberts-Thompson, I. C., Chapman, C. B., Forsyth, K. P., Kahl, L. P. and Cruise, K. M.** (1982). Analysis of infection characteristics and antiparasite immune responses in resistant compared with susceptible hosts. *Immunological Reviews* **61**, 137–188.
- Morakote, N., Sukhavat, K., Khamboonruang, C., Siriprasert, V., Suphawitayanukul, S. and Thamasonthi, W.** (1992). Persistence of IgG, IgM, and IgE antibodies in human trichinosis. *Tropical Medicine and Parasitology* **43**, 167–169.
- Müller, N. and von Allmen, N.** (2005). Recent insights into the mucosal reactions associated with *Giardia lamblia* infections. *International Journal for Parasitology* **35**, 1339–1347.
- Owen, I. L.** (2005). Parasitic zoonoses in Papua New Guinea. *Journal of Helminthology* **79**, 1–14.
- Prophet, E. D., Mills, B., Arrington, J. B. and Sobin, L. H.** (1994). *Laboratory Methods in Histotechnology of the Armed Forces Institute of Pathology* 2nd Edn. American Registry of Pathology, Washington, D.C., USA.
- Roberts-Thompson, I. C., Grove, D. I., Stevens, D. P. and Warren, K. S.** (1976). Suppression of giardiasis during the intestinal phase of trichinosis in the mouse. *Gut* **17**, 953–958.
- Rosenberg, E. B., Polmar, S. H. and Whalen, G. E.** (1971). Increased circulating IgE in trichinosis. *Annals of Internal Medicine* **75**, 575–578.
- Singer, S. M. and Nash, T. E.** (2000). T-cell-dependent control of acute *Giardia lamblia* infections in mice. *Infection and Immunity* **68**, 170–175.
- Stäger, S., Gottstein, B. and Müller, N.** (1997). Systemic and local antibody response in mice induced by a recombinant peptide fragment from *Giardia lamblia* variant surface protein (VSP) H7 produced by a *Salmonella typhimurium* vaccine strain. *International Journal for Parasitology* **27**, 965–971.
- Stäger, S. and Müller, N.** (1997). *Giardia lamblia* infections in B-cell-deficient transgenic mice. *Infection and Immunity* **65**, 3944–3946.
- Urban, J. F. Jr, Schopf, L., Morris, S. C., Orekhova, T., Madden, K. B., Betts, C. J., Gamble, H. R., Byrd, C., Donaldson, D., Else, K. and Finkelman, F. D.** (2000). Stat6 signaling promotes protective immunity against *Trichinella spiralis* through a mast cell- and T cell-dependent mechanism. *Journal of Immunology* **15**, 2046–2052.
- Venkatesan, P., Finch, R. G. and Wakelin, D.** (1997). A comparison of mucosal inflammatory responses to *Giardia muris* in resistant B10 and susceptible BALB/c mice. *Parasite Immunology* **19**, 137–143.
- Watanabe, N., Bruschi, F. and Korenaga, M.** (2005). IgE: a question of protective immunity in *Trichinella spiralis* infection. *Trends in Parasitology* **21**, 175–178.
- Zhou, P., Li, E., Zhu, N., Robertson, J., Nash, T. and Singer, S. M.** (2003). Role of interleukin-6 in the control of acute and chronic *Giardia lamblia* infections in mice. *Infection and Immunity* **71**, 1566–1568.