MICROBIAL PATHOGENESIS

Influence of bacteria from the duodenal microbiota of patients with symptomatic giardiasis on the pathogenicity of *Giardia duodenalis* in gnotoxenic mice

MARCIA F. TORRES, ANA P. T. UETANABARO*, ALESSANDRA F. COSTA, CELSO A. ALVES, LUIS M. FARIAS*, EDUARDO A. BAMBIRRA†, FRANCISCO J. PENNA, ENIO C. VIEIRA‡ and JACQUES R. NICOLI*

Departamento de Pediatria, Faculdade de Medicina, *Departamento de Microbiologia, Instituto de Ciências Biológicas, †Departamento de Anatomia Patológica, Faculdade de Medicina and ‡Departamento de Bioquímica-Imunologia, Universidade Federal de Minas Gerais, Belo Horizonte MG, Brazil

Recent studies have shown that the intestinal microbiota is essential for the pathogenicity but not for the multiplication of *Giardia duodenalis* in the intestinal lumen. The microbial components responsible for this phenomenon are not known. Twenty-eight facultative and three strictly anaerobic micro-organisms were isolated from the dominant duodenal microbiota of five patients with symptomatic giardiasis. The bacterial combinations from each patient were associated with groups (GN) of germ-free mice. Five days after the association, when their faecal populations ranged from 10^7 to 10^9 cfu/g, all groups were inoculated intragastrically with 10^5 viable trophozoites of *G. duodenalis* strain BT6. Two groups of germ-free (GF) and conventional (CV1) mice were also infected. Gnotobiotic animals were killed 10 days after infection and GF and CV1 animals were killed 10, 20 and 30 days after infection. More marked pathological alterations were detected in CV1 mice when compared with GF animals. Gnotobiotic animals showed intermediate pathological alterations between CV1 and GF mice. The CV1 and GF groups became infected by day 3 and faecal cyst levels were similar in both groups throughout the experiment. Total and *G. duodenalis*-specific IgA levels in the intestinal fluid and *G. duodenalis*-specific IgM and IgG levels in the serum increased during the infection and were higher in CV1 animals at all times tested when compared with GF mice. The present results confirm the stimulatory activity of the intestinal microbiota on the pathogenicity of *G. duodenalis*, and some combinations of microbial components of the dominant duodenal ecosystem from patients with symptomatic giardiasis can partially develop this function. However, none of these combinations was able to stimulate the protozoan pathogenicity in the same manner as the entire intestinal microbiota.

Introduction

Giardia infections result in various clinical manifestations ranging from asymptomatic or transient intestinal complaints that resolve spontaneously to severe long-standing disease with malabsorption and debilitation [1]. The proximal portion of the small intestine is the

primary site of its pathological action. The exact mechanism underlying the variable clinical symptomatology is not fully understood; however, recent studies have shown that the intestinal microbiota is indispensable for the pathogenic expression but not for the multiplication of *Giardia duodenalis* (*G. 'lamblia'*) [2]. The presence of gut microbiota is also essential for the pathogenic expression of some other enteric protozoa such as *Entamoeba histolytica* [3], *Histomonas meleagridis* [4], *Eimeria tenella* [5], *Eim. falciformis* [6], *Blastocystis hominis* [7] and *Eim. ovinoidalis* [8]. Working with *Ent. histolytica*, Mirelman and Bracha [9] reported that lesions were observed only with

Received 20 Jan. 1999; revised version accepted 25 May 1999.

Corresponding author: Dr J. R. Nicoli (e-mail: jnicoli@mono.icb.ufmg.br).

axenic amoebae re-associated with a gram-negative bacterium. The microbial components responsible for the phenomenon involving *G. duodenalis* are not known. Various hypotheses have been proposed with regard to the mechanisms involved in this pathogenic stimulation by bacteria. Some of them are related to changes caused by axenisation of the protozoa, such as a possible decrease in adhesion (changes in superficial carbohydrates) or in invasive abilities (decrease of proteolytic activities) observed in protozoa cured of their bacterial endosymbionts [10, 11]. Other explanations are related to the axenisation of the host, such as the thicker intestinal mucin layer (physical and chemical barrier) observed in germ-free animals [12]. It is unlikely that virulence can be attributed entirely to the intervention of bacterial microbiota. Immunodepressed status, host nutrition and age, and strain zymodeme are other possible important predisposing factors [1].

In this work, some bacterial components of the duodenal dominant microbiota from patients with symptomatic giardiasis were tested for their ability to stimulate *G. duodenalis* pathogenicity in gnotoxenic mice.

Patients and methods

Patients

Five children treated for symptomatic giardiasis at the Federal University of Minas Gerais Hospital were the donors of the dominant duodenal microbiota. The study was approved by the Scientific and Ethics Committee of the Hospital. The children participated in the study with the informed consent of their mothers. A duodenal biopsy was used to obtain a sample of duodenal fluid. This material was diluted in a tube containing 1 ml of pre-reduced anaerobically sterilised dilution fluid (Ringer-PRAS) flushed with oxygen-free CO₂.

Preparation of dominant duodenal microbiota

Within 1 h of collection, the diluted duodenal preparation was introduced into an anaerobic chamber (Forma Scientific, Marietta, OH, USA), containing an atmosphere of N₂ 85%, H₂ 10% and CO₂ 5%, and serially diluted in tubes containing pre-reduced buffered saline. Samples from 10¹ to 10⁶ dilutions were spread on blood agar plates supplemented with haemin and menadione (BAS) and incubated at 37°C for 7 days. Colony counts were then performed on the appropriate dilution, and morphologically different colonies were isolated. The isolates were stained by Gram's method and tested for aerotolerance.

Mice

Germ-free NIH 21-day-old mice (Taconic, Germantown, NY, USA) of both sexes were used as microbial

receptors in this study. The animals were housed in flexible plastic isolators (Class Biologically Clean, Madison, WI, USA) and handled according to established procedures [13]. The animals were fed a commercial autoclavable diet (Nuvital, Curitiba, PR, Brazil) ad libitum. Germ-free mice associated with filtered *G. muris*-free faecal microbiota, as described in a previous report [14], were used as the conventional (CV) controls. Experiments with gnotoxenic mice or mice with filtered intestinal microbiota were performed in micro-isolators (UNO Roestvaststaal BV, Zevenaar, The Netherlands).

Association of germ-free mice with duodenal isolates

The microbial isolates were grown on BAS for 48 h at 37°C in an anaerobic chamber and washed off with buffered saline. A combination of isolates from each patient was associated immediately with a group of five germ-free mice. A 0.5-ml portion of these suspensions was used for inoculation of each animal by gastric intubation to obtain the gnotoxenic (GN) mice. All the handling and inoculating operations with GN mice were done under a laminar flow hood (Veco, Campinas, SP, Brazil).

G. duodenalis infection

Five days after the association, each group of GN mice was subdivided into two groups comprising three and two animals, which were either inoculated intragastrically with 10⁵ viable trophozoites of *G. duodenalis* strain BTU6/89/JMS (kindly supplied by Dr M. I. T. Sogayar, Department of Parasitology, Paulista State University, Botucatu, SP, Brazil) or not inoculated, respectively. Two groups of 20 germ-free (GF) and 20 *G. muris*-free conventional (CV1) mice were also infected and used as controls. A third group of five non-infected *G. muris*-free conventional (CV2) mice was used as another control. Groups of five infected GF and CV1 animals were killed by ether inhalation 0, 10, 20 and 30 days after infection. All the GN and CV2 animals were killed 10 days after infection. Histopathological examination was performed on all GF, CV and GN mice.

Histopathological examination

After the mice were killed, the small bowel was removed. A 1-cm segment was excised 5, 15 and 25 cm from the gastroduodenal junction. The excised segment was opened longitudinally, oriented on filter paper and fixed in formaldehyde 4% for histopathological examination. After fixation, pieces of tissues were processed for paraffin embedding. Histopathological sections (3–5 µm) were stained with haematoxylin-eosin and PAS-Alcian Blue, pH 2.5, and the slides were examined by a single pathologist who was unaware of the experimental conditions for each group. The slides were then

coded and decoded only after the report had been written. Other histopathological sections (3 μm) were used for morphometric analysis of the surface epithelium volume and the mononuclear exudate density of the lamina propria by computerised digital image processing [15]. The measurement was performed in relation to a defined $10^4 \mu\text{m}^2$ constant area of the muscularis mucosae. The images were obtained through a microscope fitted with a colour video camera (Model 1HR; Micro-Image Video Systems, Boyertown, PA, USA) and transmitted to an image display monitor (Model PVM-1343 MD; Sony Trinitron, Japan). The digitised images were viewed on a 20" Philips monitor (1.024×768 lines of resolution) and the analysis was performed by a computer with an Intel Pentium MMX 133 processor with 16 megabytes of RAM.

Parasitological analysis

Quantification of cysts in faeces and of trophozoites in the small bowel was performed as described by Roberts-Thomson et al. [16].

Immunoglobulin determination

Total and *G. duodenalis*-specific immunoglobulins of class A in the intestinal fluid were determined by ELISA by a modification of the method described by Lim et al. [17]. In this modification, dilution of intestinal contents was performed at a ratio of 0.5 g of contents/2 ml of PBS, pH 7.2. In the original method, intestinal contents were always diluted in 2 ml of PBS independent of their weight.

Statistical analysis

Data were analysed by analysis of variance or by the Kruskal-Wallis test for variables showing a normal distribution or not, respectively. The statistical treatment of the results was performed with Statistical Analysis System software (SAS Institute, Cary, NC, USA), with the level of significance set at $p < 0.05$.

Results

Preparation and association of duodenal and faecal bacteria

Table 1 shows that 31 bacteria were isolated in the anaerobic chamber from the dominant duodenal microbiota of five patients with symptomatic giardiasis. The first patient (A) showed no bacteria in his duodenal fluid, even at 10^1 dilution. The second (B) showed only one colony morphology at a population level of $3.90 \log_{10}$ cfu/ml. In the duodenal fluid of the third patient (C), five colony morphologies were found ranging from 4.84 to $7.04 \log_{10}$ cfu/ml. The fourth (D) and fifth (E) children showed 15 and 10 different microbial morphologies, respectively, present in total population levels of c. $4.0 \log_{10}$ cfu/ml. Aerotolerance tests showed that these micro-organisms were predominantly facultative species (28 micro-organisms) with only three strict anaerobes. Three of the facultative species were yeasts. The micro-organisms were associated in the same combination as in the human donor with groups (GNB, GNC, GND and GNE) of 21-day-old germ-free mice reared in micro-isolators. Five days after association, their faecal populations ranged from 7.0 to $9.0 \log_{10}$ cfu/g when the animals were experimentally infected with *G. duodenalis*. The GF and CV1 groups were also challenged with the protozoa at the same time.

Histopathology of the small intestine of GF, GN and CV mice

Fig. 1 shows greater pathological alterations in CV1 mice (Fig. 1A) when compared with GF animals (Fig. 1B). These differences were observed throughout the time of infection, but with decreasing intensity by day 30 after infection. The principal aspects of these alterations were villus hypotrophy, high reactional hyperplasia of the lymphoid component and the presence of parasitic trophozoites on the mucosa and in the intestinal lumen. GN mice showed intermediate pathological alterations between GF and CV1 animals (Fig. 1C). No pathological changes were observed in non-infected GN or CV2 animals.

Table 1. Microbial components of the dominant duodenal microbiota from five patients with symptomatic giardiasis

Characteristic	Patients				
	A	B	C	D	E
Age	11 months	7 years	2 years 2 months	2 years	3 years 3 months
Sex	M	F	M	M	F
Total anaerobes*	<1.0	3.90	7.04	3.84	4.15
Number of components with different micromorphology	—	1	5	15	10
Number of strict anaerobes	0	0	0	2	1
Number of facultative anaerobes	0	1	5	13	9
Gram-positive organisms	0	0	0	11	7
Gram-negative organisms	0	1	5	2	2
Yeast	0	0	0	2	1

* $\log_{10}$ cfu/ml duodenal fluid.

Fig. 1. Histopathological aspect of small intestine mucosa from germ-free mice associated with *G. muris*-free intestinal microbiota (CV1, A), germ-free mice (GF, B) or germ-free mice associated with bacteria isolated from dominant duodenal microbiota of a patient with symptomatic giardiasis (GN, C). The animals were challenged intragastrically with 10^5 viable trypozites of *G. duodenalis* strain BT-6. (PAS-Alcian Blue, $\times 160$).

A computerised morphometric analysis 10 days after experimental infection is presented in Tables 2 and 3. Both GF and CV1 mice showed a significant reduction of surface epithelial volume during the infection (Table

2). By day 30 after infection, the values reached 0.11 and 0.19 (10^6) μm^3 for GF and CV1 animals, respectively (data not shown). This reduction was not observed in GN mice. There was no difference in this morphometric analysis between GF, CV1 and GN animals before the infection. Table 3 shows that the mononuclear exudate density was significantly lower in GF mice than in their CV1 and GN counterparts before infection. During infection, both GF and CV1 showed a significant increase in lamina propria cellularity, which was more intense in the latter group.

Faecal cyst counts and faecal weights of GF, GN and CV1 mice

The two groups became infected by day 3 and faecal cyst levels were similar in all CV1 and GF groups throughout the experiment (c. 10^6 cysts/g of faeces) (Fig. 2). By day 10 of infection, the same cyst levels were found in GN faeces. Faecal weight before and during the infection was higher in GF animals than in the GN and CV1 groups (data not shown).

Faecal IgA and serum IgM and IgG of GF, GN and CV1 mice

G. duodenalis-specific (Fig. 3) and total (Fig. 4) IgA levels increased in the intestinal fluid during the infection and were higher in CV animals throughout the experimental period, particularly for total IgA, than in GF mice. Table 4 shows the *G. duodenalis*-specific IgA in the intestinal fluid and the *G. duodenalis*-specific IgM and IgG in the serum from GF, GN and CV1 mice, 10 days after the oral challenge with the protozoan. An increase in these IgA, IgM and IgG levels with the challenge was observed only for the GF, CV1 and GNE groups.

Discussion

In contrast to results obtained in previous studies [18], there was no indication of duodenal microbial overgrowth in the patients with symptomatic giardiasis (Table 1). The mean microbial population levels of c. $4.0 \log_{10}$ cfu/ml of duodenal fluid, as well as minimum and maximum values ranging from 0 to $7.0 \log_{10}$ cfu/ml, can be considered normal values for the duodenum. The isolation of a higher number of facultative anaerobes was also expected, because of the relatively high oxygen level and oxidation-reduction potential in this portion of the digestive tract [19].

The pathogenesis of giardiasis may be mediated by many factors, including a giardia toxin, mechanical injury of the mucosa or synergy with other enteropathogens, as well as immune-mediated mechanisms. Some endosymbionts described for both *G. muris* and *G. duodenalis* have not been excluded from this

Table 2. Surface epithelium volume for $10^4 \mu\text{m}^2$ of small intestine muscularis mucosae from germ-free (GF), conventional (CV1) or gnotoxenic (GN) mice, 10 days after oral challenge with *G. duodenalis* or not

Groups of mice	Mean (SD) surface epithelial volume ($10^6 \mu\text{m}^3$)	
	No <i>G. duodenalis</i>	<i>G. duodenalis</i> challenge
GF	0.26 (0.02)*	0.17 (0.01) [†]
CV1	0.29 (0.04)*	0.24 (0.05) [‡]
GN		
B	ND	0.13 (0.01)
C	0.28 (0.03)*	0.26 (0.02) [‡]
D	0.31 (0.00)*	0.32 (0.20) [‡]
E	0.26 (0.07)*	0.35 (0.04) [‡]

ND, not determined.

*,[†],[‡]Significantly different from groups with different microbiological status (GF, CV1 or different GN), but with the same infected status (challenged or not with *G. duodenalis*), ANOVA.

[†]Significantly different from its non-infected counterpart with the same microbiological status (GF, CV1 or different GN), ANOVA.

Table 3. Mononuclear exudate density of the lamina propria for $10^4 \mu\text{m}^2$ of small intestine muscularis mucosae from germ-free (GF), conventional (CV1) or gnotoxenic (GN) mice, 10 days after an oral challenge with *G. duodenalis* or not

Groups of mice	Mean (SD) mononuclear exudate density ($10^6 \mu\text{m}^3$)	
	No <i>G. duodenalis</i>	<i>G. duodenalis</i> challenge
GF	0.91 (0.14)*	1.03 (0.15)*
CV1	2.25 (0.17) [†]	3.10 (0.10) ^{†§}
GN		
B	ND	2.83 (0.21)
C	2.10 (0.27) ^{†‡}	2.32 (0.24) [‡]
D	2.59 (0.47) [†]	1.72 (0.24)
E	1.74 (0.27) [‡]	2.47 (0.19) ^{‡§}

ND, not determined.

*,[†],[‡],^{||}Significantly different from groups with different microbiological status (GF, CV1 or different GN), but with the same infected status (challenged or not with *G. duodenalis*), ANOVA.

[§]Significantly different from its non-infected counterpart with the same microbiological status (GF, CV1 or different GN), ANOVA.

Fig. 2. Cyst number of faeces from germ-free (GF) or conventional (CV1) mice infected experimentally with 10^5 viable trophozoites of *G. duodenalis* strain BT-6.

Fig. 3. *G. duodenalis*-specific IgA in intestinal fluid from germ-free (GF) or conventional (CV1) mice infected experimentally with 10^5 viable trophozoites of *G. duodenalis* strain BT-6.

correlation. Greater pathological alterations observed in CV1 mice when compared with GF animals (Fig. 1A and B) confirmed the results of Torres et al. [2]. However, the histopathological differences between GF and CV1 animals seemed to decrease during the course of infection. The results of the present study suggest

that the bacteria responsible for part of the stimulation of *G. duodenalis* pathogenicity are present in the dominant duodenal microbiota. However, none of the individual or combined micro-organisms was able to stimulate the pathogenicity of *G. duodenalis* to the levels observed in CV1 mice. Other components of the

Fig. 4. Total IgA in intestinal fluid from germ-free (GF) or conventional (CV1) mice infected experimentally with 10^5 viable trophozoites of *G. duodenalis* strain BT-6.

subdominant and residual intestinal microbiota may be important for the phenomenon (e.g., secondary pathological insult). Another possible factor explaining the different pathological stimulation between GN and CV1 animals could be the age of association used in the present study. CV1 mice were colonised at birth by their normal microbiota instead of via an experimental microbial association performed soon after weaning for GN animals when the physiological and immunological status of the mice was different.

In the mouse model, a reduction of the jejunal villus:crypt ratio occurs simultaneously with an impaired weight gain [16]. In the present study, a progressive reduction of surface epithelium volume was found during experimental infection with *G. duodenalis* and in the absence of any bacterial component (GF). The bacterial association (GN or CV1) did not lead to a more pronounced reduction.

This implies that other physiological mechanisms provoke this morphological phenomenon. Immunological events are probably the most likely explanation. The challenge with *G. duodenalis* increased the number of mononuclear cells in the lamina propria of both GF and CV1 animals, and this stimulation was 90% greater in the latter. Several defence mechanisms function on the surface of the intestinal mucosa; some are non-immunological in nature, while others, such as secretory IgA production, are clearly antigen-driven. In addition to local humoral defence, T-cell-mediated responses also provide some degree of protection at this interface. Intra-epithelial lymphocytes (IELs) are definitively influenced by intraluminal antigens, as is evident from observations of the fetal intestine at weaning, and in adult enteropathies such as cryptosporidiosis, tropical enteric diseases and giardiasis [20]. This issue has become more complex in recent years with the discovery that IELs expressing the $\lambda\delta$ for the T-cell receptor (TCR) are also influenced by, and possibly involved in, these pathological processes. den Hollander et al. [21] also suggest that IELs have a potential protective role during giardiasis, as well as a simultaneous pathogenic effect.

The results of cyst appearance and levels in GF and CV1 mice experimentally infected with *G. duodenalis* were similar to those previously obtained by Torres et al. [2] and confirmed that the normal digestive microbiota was not necessary for protozoan multiplication. Similar results were obtained with other pathogenic protozoa such as *Ent. histolytica* [3], *Eim. tenella* [5], *Eim. falciformis* [6] and *Eim. ovinoidalis* [8]. The higher faecal weight of GF mice when compared with their CV1 counterparts could be explained by the well documented water loss observed in GF rodents [12].

Table 4. *G. duodenalis*-specific IgA in the intestinal fluid and IgM and IgG levels in the serum from germ-free (GF), conventional (CV1) or gnotoxenic (GN) mice, 10 days after an oral challenge with *G. duodenalis* or not

Groups of mice	Challenge with <i>G. duodenalis</i>	Mean (SD) OD ₄₉₂ value		
		IgA	IgM	IgG
GF	–	0.024 (0.018)	0.048 (0.040)	0.011 (0.005)
	+	0.138 (0.019)*§	0.170 (0.070)*§	0.088 (0.013)*§
CV1	–	0.078 (0.003)	0.277 (0.047)	0.036 (0.017)
	+	0.410 (0.115)†§	0.600 (0.050)†§	0.155 (0.038)*§
GN	–	ND	ND	ND
	+	ND	0.415 (0.063)	0.105 (0.025)
	–	0.155 (0.115)	0.780 (0.012)	0.099 (0.007)
	+	0.160 (0.044)*‡	0.558 (0.130)†	0.049 (0.050)
	–	0.278 (0.006)	0.305 (0.136)	0.015 (0.005)
	+	0.252 (0.044)‡	0.193 (0.036)*	0.010 (0.004)
E	–	0.170 (0.048)	0.324 (0.043)	0.037 (0.002)
	+	0.424 (0.178)†	0.543 (0.023)†	0.071 (0.001)

ND, not determined.

*†‡§|| Significantly different from groups with different microbiological status (GF, CV1 or different GN), but with the same infected status (challenged or not with *G. duodenalis*), Kruskal-Wallis test.

§ Significantly different from its non-infected counterpart with the same microbiological status (GF, CV1 or different GN), Kruskal-Wallis test.

In spite of their underdeveloped immunological system, GF animals did not develop intestinal pathological alterations (although their CV counterparts did) during experimental infection with *G. duodenalis*. This fact reinforces the importance of bacterial components from the intestinal microbiota as stimulatory factors for protozoan pathogenicity. The greater increase in total IgA compared with the *G. duodenalis*-specific IgA may have been due to a secondary pathological insult from components of the intestinal microbiota.

The present study showed that micro-organisms isolated from the dominant duodenal microbiota of patients with symptomatic giardiasis can stimulate the pathogenicity of *G. duodenalis* in a gnotobiotic animal model.

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Financiadora de Estudos (FINEP) e Projetos and Pró-Reitoria de Pesquisa, UFMG (PRPq-UFMG). We thank João da Costa Viana (Department of Parasitology, ICB-UFMG, Belo Horizonte, MG, Brazil) and Maria Gorete Barbosa Ribas for valuable technical assistance.

References

1. Thompson RCA, Reynoldson JA, Mendis AHW. Giardia and giardiasis. *Adv Parasitol* 1996; 32: 71–160.
2. Torres MRF, Silva MEC, Vieira EC et al. Intra-gastric infection of conventional and germfree mice with *Giardia lamblia*. *Braz J Med Biol Res* 1992; 25: 349–352.
3. Phillips BP, Wolfe PA, Rees CW, Gordon HA, Wright WH, Reyniers JA. Studies on the ameba–bacteria relationship in amebiasis. Comparative results of the intracecal inoculation of germfree, monocontaminated, and conventional guinea pigs with *Entamoeba histolytica*. *Am J Trop Med Hyg* 1995; 4: 675–692.
4. Franker CK, Doll JP. Experimental histomoniasis in gnotobiotic turkeys. II. Effects of some cecal bacteria on pathogenesis. *J Parasitol* 1964; 50: 636–640.
5. Visco RJ, Burns WC. *Eimeria tenella* in bacteria-free and conventionalized chicks. *J Parasitol* 1972; 58: 323–331.
6. Owen D. *Eimeria falciformis* (Eimer, 1870) in specific pathogen free and gnotobiotic mice. *Parasitology* 1975; 71: 293–303.
7. Phillips BP, Zierdt CH. Blastocystis hominis: pathogenic potential in human patients and in gnotobiotics. *Exp Parasitol* 1976; 39: 358–364.
8. Gouet P, Yvore P, Naciri M, Contrepoint M. Influence of digestive microflora on parasite development and the pathogenic effect of *Eimeria ovinoidalis* in the axenic, gnotoxenic and conventional lamb. *Res Vet Sci* 1984; 36: 21–23.
9. Mirelman D, Bracha R. Adherence and ingestion of bacteria by trophozoites of *Entamoeba histolytica*. *Arch Invest Med* 1982; 13 Suppl 3: 109–122.
10. Phillips BP. *Entamoeba histolytica*: concurrent irreversible loss of infectivity pathogenicity and encystment potential after prolonged maintenance in axenic culture in vitro. *Exp Parasitol* 1973; 34: 163–167.
11. Dwyer DM, Chang K-P. Surface membrane carbohydrate alterations of a flagellated protozoan mediated by bacterial endosymbionts. *Proc Natl Acad Sci USA* 1976; 73: 852–856.
12. Wostmann BS, Bruckner-Kardoss F. Functional characteristics of gnotobiotic rodents. In: Sasaki S, Ozawa A, Hashimoto K (eds) Recent advances in germfree research. Tokyo, Tokai University Press. 1981: 321–325.
13. Pleasants JR. Gnotobiotics. In: Melby EC, Altman NH (eds) Handbook of laboratory animal science. Cleveland, CRC Press. 1974: 119–173.
14. Torres MF, Uetanabaro AP, Bambirra EA, Pena FJ, Vieira EC, Nicoli JR. Obtaining mice associated with a filtered intestinal microbiota free for *Giardia muris* and its use as an animal model for experimental infection with *Giardia lamblia*. In: Hashimoto K, Sakakibara B, Tazume S, Shimizu K (eds) Germfree life and its ramifications. Shiozawa, Sense Printing. 1996: 127–129.
15. Puglisi RN, Whalen TV, Doolin EJ. Computer analyzed histology of ischemic injury to the gut. *J Pediatr Surg* 1995; 30: 839–844.
16. Roberts-Thomson IC, Stevens DP, Mahmoud AAF, Warren KS. Giardiasis in the mouse: an animal model. *Gastroenterology* 1976; 71: 57–61.
17. Lim TS, Messih N, Watson RR. Immune components of the intestinal mucosae of ageing and protein deficient mice. *Immunology* 1981; 43: 401–407.
18. Adam RD. The biology of *Giardia* spp. *Microbiol Rev* 1991; 55: 706–732.
19. Finegold SM, Sutter VL, Mathisen GE. Normal indigenous intestinal flora. In: Hentges DJ (ed) Human intestinal microflora in health and disease. New York, Academic Press. 1983: 3–31.
20. Marsh MN, Cummins AG. The interactive role of mucosal T lymphocytes in intestinal growth, development and enteropathy. *J Gastroenterol Hepatol* 1993; 8: 270–278.
21. denHollander N, Riley D, Befus D. Immunology of giardiasis. *Parasitol Today* 1988; 4: 124–131.