RELATÓRIO TÉCNICO-CIENTÍFICO

Ref: CBS '1070/96- "Antagonismo contra bactérias enteropatogênicas por substâncias inibidoras difusíveis produzidas pela microbiota gastrointestinal"

O relatório corresponde às atividades científicas relativas a 1997 e 1998. O projeto enquadrou-se dentro das linhas de trabalho do Laboratório de Ecologia e Fisiologia de Microorganismos do Departamento de Microbiologia, ICB - UFMG que têm como objetivo estudar as funções, em particular de proteção ecológica, dos componentes da microbiota gastrointestinal normal. As possibilidades de preservação, reforço ou compensação dessas funções são também estudados.

A seguir, encontraram-se cópias dos trabalhos publicados no período onde o nome da FAPEMIG foi citado nos agradecimentos para o suporte financeiro. Em anexo, encontra-se também a lista dos trabalhos apresentados em congressos e teses ou dissertações defendidas no período de 1997 e 1998 e para os quais o apoio da FAPEMIG foi de uma maneira ou outra importante.


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Resumos publicados em revistas


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50 - Antagonismo contra bactérias anaeróbias estritas e facultativas por uma substância difusível produzida por um Lactobacillus sp isolado da microbiota fecal de rat. Santos ARM, Nardi RD, Nicolli JR, Farias LM, Carvalho MAR, Bandeiras L, Santoro MM. VII Encontro de Pesquisa, 09-13/11/98, Belo Horizonte MG. Resumo p. 82.


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Intracellular Signal Triggered by Cholera Toxin in Saccharomyces boulardii and Saccharomyces cerevisiae

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As is the case for Saccharomyces boulardii, Saccharomyces cerevisiae W303 protects Fisher rats against cholera toxin (CT). The addition of glucose or dinitrophenol to cells of S. boulardii grown on a nonfermentable carbon source activated trehalase in a manner similar to that observed for S. cerevisiae. The addition of CT to the same cells also resulted in trehalase activation. Experiments performed separately on the A and B subunits of CT showed that both are necessary for activation. Similarly, the addition of CT but not its separate subunits led to a cyclic AMP (cAMP) signal in both S. boulardii and S. cerevisiae. These data suggest that trehalase stimulation by CT probably occurred through the cAMP-mediated protein phosphorylation cascade. The requirement of CT subunit B for both the cAMP signal and trehalase activation indicates the presence of a specific receptor on the yeast able to bind to the toxin, a situation similar to that observed for mammalian cells. This hypothesis was reinforced by experiments with 125I-labeled CT showing specific binding of the toxin to yeast cells. The adhesion of CT to a receptor on the yeast surface through the B subunit and internalization of the A subunit (necessary for the cAMP signal and trehalase activation) could be one more mechanism explaining protection against the toxin observed for rats treated with yeasts.

Viable yeast cells were recently used to improve the resistance of the intestinal ecosystem to bacterial infection (4, 16). The nonpathogenic yeast Saccharomyces boulardii (12), which is widely used in many countries for the treatment of antibiotic-induced gastrointestinal disorders (18) and Clostridium difficile-associated enterocolitis (2), has been extensively studied. Controlled clinical trials have also demonstrated this activity in the treatment of various types of enteric syndromes, such as acute infantile gastroenteritis (3) and diarrhea associated with continuous-flow enteral nutrition (17).

Several possible mechanisms for the protective effect of S. boulardii against infections by either indigenous gastrointestinal microflora or recently acquired exogenous microbes (1) have been proposed, mainly based on results from studies with experimental animals. One of these hypotheses is related to the inhibition of bacterial toxin production or action. Experimentally, S. boulardii inhibits C. difficile toxin A binding and enterotoxicity in rat ileum (14). The same yeast also inhibits or neutralizes the enteroxotoxicity of Escherichia coli toxins and Vibrio cholerae toxin (7, 11, 23). Recent results have shown that S. boulardii produces a 120-kDa protein able to neutralize the effect of cholera toxin (CT) (6). The mechanism of this toxin-neutralizing effect may be related to the ability of a protein from the yeast to bind to a receptor that in turn regulates intracellular adenylate cyclase levels. An additional mechanism may be specific adhesion of the toxin to the yeast.

The 84-kDa V. cholerae toxin, which is functionally, structurally, and immunologically similar to E. coli heat-labile enterotoxin, is composed of the catalytically active A subunit and five identical B subunits that constitute the binding region of the toxin. Binding of the CT to ganglioside receptors (GM1) of enterocyte microvilli is followed by the internalization of subunit A, which catalyzes the activation of adenylate cyclase, causing a rise in cyclic AMP (cAMP) levels that triggers active secretion of chloride and bicarbonate in crypt cells and inhibits chloride absorption in the villi. Since water flows passively with electrolytes in response to osmotic gradients, CT causes the cessation of the absorption of water through villi and the amplification of water secretion from crypt cells, resulting in copious diarrhea (9).

In the yeast Saccharomyces cerevisiae, the addition of rapidly fermented sugar to cells growing on a nonfermentable carbon source is known to trigger a cAMP-mediated protein phosphorylation cascade. The addition of glucose causes a rapid, transient increase in cAMP levels, followed by the activation of trehalase and other enzymes known to be regulated by cAMP-dependent protein phosphorylation. The effect of glucose can be mimicked by the addition of protonophores such as dinitrophenol (DNP) at a low external pH. The normal physiological function of the glucose-induced cAMP-mediated protein phosphorylation cascade is to switch metabolism from gluconeogenic-respiratory to fermentative (22).

In the present study, we hypothesized that CT is able to adhere specifically to the surfaces of different yeasts, and we assessed in vivo some of the biochemical effects of this adhesion on microorganisms.

MATERIALS AND METHODS

Animals and treatments. Litters of male Fisher rats weighing about 40 g (Department of Nutrition, Federal University of Ouro Preto, Ouro Preto, Brazil) were used. The animals were divided at random into experimental and control groups just after weaning. S. cerevisiae W303 production was carried out with a bench-top fermentor (model MF 115, New Brunswick Scientific Co., Edison,
N.I.). During the operation, aeration, agitation, and temperature were adjusted to 1 volume of air per volume of medium per min (vvm), 600 rpm, and 30°C, respectively. Stationary-phase cells were harvested by centrifugation and thoroughly washed with distilled water. The biomass was resuspended in saline to obtain about 2 x 10^7 CFU/ml, and aliquots of 0.5 ml were administered to the experimental group animals by gastric gavage three times a day. The animals in the control group received saline according to the same schedule. Five days after the beginning of these treatments, an 18-h culture of V. cholerae (recently isolated from a clinical case at Fundação Enec procurada, Belo Horizonte, Brazil) that had been incubated at 37°C in brain heart infusion (10^8 CFU/ml) was inoculated by gastric intubation into both experimental and control group animals. After the bacterial challenge, treatments with yeast suspension or saline were continued for an additional 5 days. By days 2 and 5 of infection (corresponding to 7 and 10 days of treatments, respectively), five animals from each group were sacrificed by ether inhalation. Liver, spleen, mesenteric lymph node, small intestine (upper, middle, and lower), and colon (middle) samples were obtained and fixed in 10% neutral formalin. The fix tissues were dehydrated, embedded in paraffin, cut into 7-µm-thick sections, and stained by the routine hematoxylin and eosin method. The slides were deparaffinized and examined by only one pathologist, who did not have access to the experimental conditions for each group. After the reports had been written, the material was declassified.

**Yeast strains and growth conditions.** S. boulardii (Florant; Merck S.A., Rio de Janeiro, Brazil) and S. cerevisiae W303 strains were grown in a rotary incubator (200 rpm) at 30°C in YPG medium, containing 1% (wt/vol) yeast extract, 2% (wt/vol) peptone, and 3% (wt/vol) glucose. Cells in the logarithmic phase were harvested by centrifugation at 3,000 x g for 5 min. Three times with distilled water, and resuspended in 10 mM morpholineethanesulfonic acid-KOH buffer (pH 6.0). The cell concentration was 20 mg (wet mass)/ml in all experiments.

**Incubation and extraction conditions.** For measurement of trehalase activity and intracellular cAMP levels, the cells were incubated in a shaking water bath at 30°C. Two samples were taken at 15-min intervals for the determination of basal trehalase activity and intracellular cAMP levels before the addition of 100 mM glucose, 2 mM DNP, different toxin concentrations, or toxin subunits A and B (Sigma Chemical Co., St. Louis, Mo.). Cells and crude extract preparations were sampled by the method of Thevelein and Bulles (20).

**Determination of trehalase activity and cAMP and protein concentrations.** Trehalase activity and cAMP and protein concentrations in crude extracts were determined as described by Thevelein and Jones (19), Thevelein et al. (21), and Lowry et al. (10), respectively. All experiments were performed at least twice, with consistent results. Representative results are shown.

**Specific binding of 125I-labeled CT to yeast cells.** The CT was iodinated by the chloramine-T method according to Cantuescos (5) with some modifications. Free 125I was separated by chromatography on a 3-mL Sephadex G-50 column. The total radioactivity incorporated into protein was determined with a gamma counter after paper chromatography (Whatman no. 1) by use of methanol saturated with KI.

S. boulardii cells (10^8 cells/ml) were incubated for 30 min with 0.1 ml 125I-labeled CT (3.6 Ci/mmol) at room temperature in a mixture containing 500 ml of 140 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO_4, 1.8 mM CaCl_2, 10 mM glucose, 25 mM HEPES (pH 7.4), and 0.02% bovine serum albumin. Bound and free radiolabeled ligands were separated after incubation by centrifugation at 12,000 x g for 10 min in an Eppendorf Microfuge. The pellets were collected and washed two times with 1 ml of cold buffer (same composition as the mixture described above but with 0.5% albumin), and the radioactivity was measured by gamma counting. Nonspecific binding was determined by incubation of the yeast cells with an excess of unlabeled CT (0.1 µM) before addition of the iodinated protein. Total and nonspecific binding experiments were done in triplicate. Specific binding indicates the amount of 125I-CT which could be displaced by unlabeled CT and was calculated as the difference between total and nonspecific binding values.

**RESULTS**

After challenge with V. cholerae, more severe histological changes were observed in animals not treated with S. cerevisiae W303 than in treated rats. Greater alterations were found in the small intestine than in other organs, i.e., severe degenerative and necrotic changes in the superficial epithelium, with karyorrhectic cells being found in the lumen. Significant reductions in the lymphoid component of the lamina propria and in mitotic activity were also noted in all portions of the small intestine. In treated animals, histological evidence of damage was minimal or absent, and a marked expansion of the lymphoid component of the intestinal lamina propria was observed.

**FIG. 1.** Effects of 1.5 and 3.0 µg of CT per ml (A), of the A or B subunit of CT (B), or of 100 mM glucose and 2 mM DNP (C) on trehalase activity in S. boulardii. Specific activity is expressed as nanomoles of glucose released per minute per milligram of protein. Asterisks indicate the time of addition of the tested compounds. Asterisks indicate significant differences for trehalase activities after the addition of CT (1.5 and 3.8 ng/ml) subunit (A and B), 100 mM glucose, or 2 mM DNP in comparison with basal trehalase activities (P < 0.05). a's indicate significant differences for trehalase activities after the addition of CT subunit A in comparison with values after the addition of CT (1.5 µg/ml) and for the same incubation time (P < 0.05).

The results in Fig. 1 and 2 show that the addition of glucose or DNP to S. boulardii cells grown on a nonfermentable carbon source caused trehalase activation (Fig. 1C) in a manner similar to that observed for S. cerevisiae W303 (Fig. 2C). On the other hand, the addition of CT to S. boulardii or S. cerevisiae W303 cells obtained under the same conditions also resulted in the stimulation of trehalase activity (Fig. 1A and 2A). Greater
stimulation of trehalase activity by CT was observed in *S. boulardii* than in *S. cerevisiae* W303 (*P* < 0.05). The separate addition of CT subunit A or B to *S. boulardii* or *S. cerevisiae* W303 cells did not cause trehalase activation (*P* > 0.05) (Fig. 1B and 2B).

The addition of glucose, DNP, or CT to *S. boulardii* or *S. cerevisiae* W303 cells induced a transient increase in the cAMP level (Fig. 3 and 4). However, this increase in the cAMP level was observed at different times, depending on the inducing agent added (0 to 5 min for glucose and DNP or 15 to 20 min for CT). As was observed for trehalase stimulation, a greater transient increase in cAMP levels was induced by CT in *S. boulardii* than in *S. cerevisiae* W303. cAMP levels were not altered when subunit A or B was added individually (Fig. 3C and 4C).

When CT was iodinated, about 60% of the radioactivity was incorporated into protein, and the specific radioactivity of the toxin was 3.4 Ci/mmol. Binding to the Eppendorf tubes in the
be used for the treatment of cataric disorders (4, 16). The pharmacodynamics of *S. boulardii* involve three different hypothetical aspects: (i) a direct antagonistic effect (15); (ii) a trophic effect, with stimulation of enzymatic expression (2) and of intestinal defense mechanisms (12, 13); and (iii) an antisecretory effect, with action on the binding of toxins to intestinal receptors. As an example of the last aspect, *S. boulardii* significantly reduced the liquid secretion and permeability for mannitol caused by toxin A of *C. difficile* in rat ileum (compared with a control group). This effect could be explained by the production of a 54-kDa protease which digested both toxin A and its receptor on enterocytes in vitro (14). *S. boulardii* also produces a 120-kDa protein which does not have proteolytic activity and which reduces the formation of cAMP by intestinal cells in a medium to which CT or *E. coli* thermolabile toxin has been added (6). Specific toxin adhesion to the yeast surface may be another mechanism responsible for this phenomenon; this mechanism was proposed in this work with CT as a toxin model. If specific receptors for CT existed on the *S. boulardii* membrane, they probably would be structurally and functionally similar to the enterocyte GM1 system. In this case, CT fixation would trigger the same intracellular signal as for enterocytes through the cAMP-mediated protein phosphorylation cascade. As a consequence, stimulation of cAMP-dependent enzymatic systems, such as trehalase, would be expected. If this mechanism is present in *S. boulardii*, it should be found in other yeasts, such as *S. cerevisiae*.

The protective effect of *S. boulardii* treatment against CT in rats (7) was also observed when *S. cerevisiae* W303 was used. These experimental results, taken together with clinical data (4, 16), suggest that this protective property is shared by yeasts in general.

The addition of CT to *S. boulardii* and *S. cerevisiae* W303 cells resulted in trehalase activation (Fig. 1A and 2A) and a cAMP signal (Fig. 3C and 4C). Moreover, this induction depended on CT integrity. The requirement of subunit B could be explained by the presence in the yeasts of a specific receptor able to bind the toxin, i.e., a situation similar to that observed in mammalian cells. Preliminary data obtained with 125I-labeled CT demonstrated the presence of a specific binding site on yeast cell surfaces. This similarity of CT-specific binding on mammalian and yeast cells was also reinforced by the observation of a kinetically correlated delay in both trehalase activation and cAMP signal induction by CT in comparison with glucose and DNP. For intestinal epithelial cells and after CT binding, there is also a lag of 15 to 60 min before adenylate cyclase is activated. This time lag is necessary to allow the A1 peptide to translocate through the membrane and come into contact with the G proteins (8). Higher levels of trehalase activity stimulation and higher cAMP levels triggered by CT in *S. boulardii* than in *S. cerevisiae* may be related to more numerous or active CT receptors on the surface of the former yeast. If such a situation is confirmed, more efficient CT inactivation by *S. boulardii* would be expected.

Taken together, these results suggest that CT (and probably other toxins) could be neutralized by binding to the yeast surface when the A subunit is internalized, triggering the stimulation of different biochemical systems, such as a cAMP signal and trehalase activity. The data show that trehalase stimulation by CT probably occurs through part of the cAMP-mediated protein phosphorylation cascade. The surface receptor for CT and the biochemical pathway for its activation of trehalase in yeasts are currently being investigated in our laboratories.

absence of yeast cells was less than 2% of the assay values. Specific binding of CT represented 48% of total binding.

**DISCUSSION**

Although the antiadhesive properties of *S. boulardii* are widely recognized, this yeast has been prescribed on an empirical basis, and the exact mechanism of its protective effect is unknown. Recent results have shown that other yeasts can also

**FIG. 4.** cAMP signalling induced by 100 mM glucose (A), by 2 mM DNP (B), or by 1.5 μg of CT or of the A or B subunit of CT per ml (C) in *S. cerevisiae* W303.
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BACTERIAL PATHOGENICITY

Ultrastructure of *Helicobacter trogontum* in culture and in the gastrointestinal tract of gnotobiotic mice

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*Helicobacter trogontum* is a micro-aerophilic urease-positive bacterium that has recently been isolated from the intestinal mucosa of rats. The purpose of this investigation was to study the ultrastructural details of this micro-organism in both pure culture and in the gastrointestinal tract of germ-free mice infected with *H. trogontum*. The micro-organism was a fusiform to slightly spiral gram-negative cell, 4–6 µm long and 0.6–0.7 µm wide, with four to seven bipolar sheathed flagella. The cytoplasm presented several irregular and also globular granules. On each side of the polar regions of the cells, there was a highly electron-dense band, the 'polar membrane'. Coccolid forms were seen in old cultures. *H. trogontum* showed several ultrastructural characteristics of the *Helicobacter* genus and much resemblance to *H. rappini* and *H. bilis*. *H. trogontum* mainly colonised the large bowel of the gnotobiotic mice where it could be seen in the lumen and also inside the enterocytes. Vacuolation of the ileal epithelial cells, loss of microvilli and pronounced desquamation of the enterocytes of the caecum were observed in the bowel colonised by the bacterium. These observations raise the possibility that *H. trogontum* could cause some harm to the host at least in particular circumstances such as when it colonises the gastrointestinal tract of a germ-free host.

Introduction

*Helicobacter pylori* is a curved, gram-negative bacterium that colonises the gastric mucosa of man [1]. Currently, *H. pylori* is implicated as an agent of chronic gastritis and peptic ulcer disease and also as an important factor in the pathogenesis of gastric adenocarcinoma and MALT lymphoma [2–5]. Since the discovery of this micro-organism the genus *Helicobacter* has been expanding rapidly and, because of the interest in understanding the association between *H. pylori* and gastroduodenal disease, other members of the genus have received increasing attention in comparative studies. For rodents, seven *Helicobacter* species have been identified. *H. muridarum* [6], *H. hepaticus* [7], *H. bilis* [8], *Flexispira rappini* (that has been reclassified as a member of the genus *Helicobacter* [9]), *H. cholecysti* [10] and, more recently, *H. trogontum* [11] and *H. rodentium* [12]. There is also a report suggesting that the human pathogen *H. cinaedi* is a natural inhabitant of the intestinal tract of hamsters [13].

Unlike *H. pylori* and some other gastric helicobacters, the species observed in rodents seem not to cause disease when they are in their natural habitats and they are considered part of the normal microbiota of the intestine, although some of them can be related to disease when found at other sites in the host [7, 8, 14, 15].

Morphological studies of new *Helicobacter* spp. reveal novel and also common structures of the genus, providing clues about what features are likely to be involved in the colonisation of the gastrointestinal mucosa and in the causation of disease [16].

*H. trogontum* has been described recently and there is little information about the ultrastructure of this organism [11]. Therefore, the purpose of this investigation was to study the ultrastructural details of *H. trogontum* in pure culture and in the gastrointestinal

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tract of germ-free mice infected with the micro-organism.

Materials and methods

In-vitro study

The type strain of \textit{H. trogontum} (ATCC 700114) was grown on sheep blood agar 10% for 1–7 days at 37°C in micro-aerophilic conditions.

Negative staining. Colonies from a 48-h culture were transferred to a 10 mM Tris-HCl buffer, pH 7.4, to obtain a heavy suspension of bacteria. One drop of the mixture was applied to a formvar-coated 300-mesh copper grid for 1 min. Excess fluid was removed and replaced with a drop of phosphotungstic acid 1% w/v, pH 6.5, for 20–30 s and examined with a Zeiss EM 10.

Ultra-thin sections. After incubation for 1, 2, 3, 5 and 7 days, the colonies were harvested from plates, separately transferred to saline 0.85% to obtain a heavy mixture and centrifuged at low speed. The pellets obtained were fixed in glutaraldehyde 2.5% v/v buffered with 0.1 M sodium cacodylate, pH 7.2, for 1 h at room temperature and gently washed three times (10 min each) in sodium cacodylate 0.1%. The pellets were then carefully included in agar 2%, post-fixed in osmium tetroxide 1% w/v buffered with 0.1 M sodium cacodylate, pH 7.2, for 40 min at room temperature, washed with 0.1 M sodium cacodylate and dehydrated in a graded alcohol series for Epon embedding. Ultra-thin sections were collected on 300-mesh copper grids, stained with uranyl acetate 5% w/v for 10 min and lead citrate for 5 min and examined with a Zeiss EM 10.

In-vivo study

Three 4-week-old germ-free mice (NIH, Taconic, Germantown, USA) were inoculated orally with a heavy suspension of the type strain of \textit{H. trogontum} (ATCC 700114) and maintained in micro-isolator cages under sterile conditions for 3 weeks, after which they were killed by spinal dislocation. Fragments from the antral and oxyntic mucosa of the stomach and from the mucosa of the duodenum, jejunum, ileum, caecum and colon were washed separately in sterile saline 0.85% for microbiological and ultrastructural studies.

Microbiological evaluation. Two mucosal fragments from each region of the stomach and of the intestine were employed for the urease test and for carbol fuchsin-stained smears. One specimen was smeared on to a glass slide, heat-fixed, stained with carbol fuchsin and examined under oil immersion lens for the presence of fusiform to slightly spiral bacteria. For the urease test, the specimen was inserted into Christensen's urea agar. The test was considered positive when the colour of the medium changed from amber to pink within 12 h.

Ultrastructural evaluation. Fragments from each region of the gastric and intestinal mucosa were immediately fixed in glutaraldehyde 2.5% v/v buffered with 0.1 M sodium cacodylate, pH 7.2, for 3 h at room temperature, and placed in three changes of 0.1 M sodium cacodylate, pH 7.2, for 10 min each. The fragments that were considered to be \textit{H. trogontum}-positive by the two microbiological tests were processed as described above for ultra-thin sections of \textit{H. trogontum} culture.

Fragments from each region of the gastric and of the intestinal mucosa of non-infected germ-free mice were also employed for ultrastructural evaluation.

Results

In-vitro study

Light microscopic examination of carbol fuchsin-stained smears from 24–48-h cultures of \textit{H. trogontum} showed fusiform to slightly curved cells with granules that appeared larger than the width of the organism. These granules showed metachromasia when stained with toluidine blue. After incubation for 3 days the granules were found less frequently and there were some coccoidal cells. Colonies of 5–7-day-old cultures showed almost exclusively coccoidal forms.

Electron microscopic examination showed that the morphology of \textit{H. trogontum} resembled that of \textit{H. rappini} and \textit{H. bilis}. In negatively stained preparations, \textit{H. trogontum} was seen as a fusiform to a slightly curved cell, 4–6 μm long and 0.6–0.7 μm wide, with four to seven sheathed flagella at both ends and periplasmic fibres that were coiled around the length of the cell giving a criss-cross appearance to the bacterium (Fig. 1).

Ultra-thin sections of the micro-organism showed the typical ultrastructure of gram-negative organisms with an outer membrane and a plasma membrane separated by a periplasmic space. The organism was slightly tapered towards the ends of the cell and the sites of the insertion of flagella were flattened (Fig. 2A). On each side of the polar regions of the cells, there was a highly electron-dense band, the 'polar membrane', that was closely adherent to the plasma membrane (Fig. 2B). The cytoplasm was granular with a large number of ribosomes, several irregular and also spherical granules (Fig. 2B and C). These granules were seen more frequently in 24–48-h cultures.

Dividing cells were easily seen in cultures incubated for 24–48 h. Atypical or coccoidal forms of \textit{H. trogontum} were seen in 4-day-old or older cultures (Fig. 2D).

In-vivo study

The microbiological tests were negative in the fragments obtained from the stomach of the infected
mice. Although the urease test was positive with samples from the duodenum and jejunum of the animals, it was not possible to detect either bacteria or any apparent cellular alteration in these regions of the bowel by electron microscopy.

In contrast, although bacteria were only rarely present in the ileum, a large number of vacuoles were observed in the epithelial cells (Fig. 3A and B). In the caecum, numerous bacteria were seen both in the lumen (Fig. 4A) and inside the enterocytes where they were observed isolated or mostly in clusters in the cytoplasm (Fig. 4B). The clusters consisted of a variable number of bacteria and were frequently localised near the nucleus of the cells (Figs. 4B and 5A). Also, pronounced desquamation and loss of microvilli were observed in this region in infected animals (Fig. 5A, B and C). Structures of different sizes and densities were consistently seen in the cytoplasm of the epithelial cells of the ileum, caecum and colon (Figs. 3A, 4B and 5). Bacteria were infrequently observed in the colon, where less evident alterations were found (Fig. 5D).

Discussion

As the Helicobacter genus has been defined primarily on genotypic rather than on phenotypic grounds, it comprises species that are ultrastructurally very diverse [16]. Some of the characteristics that are shared by almost all members of the genus are the typical cell wall structure of gram-negative bacteria, the cytoplasm with intracellular granules, the presence of polar sheathed flagella, the ‘polar membrane’ in the terminal region of the organism that seems to be related to energy production for movement, and alteration to the coccoidal form in old cultures [16]. In addition to these features, H. trogontum has an arrangement of periplasmic fibres and bipolar tufts of sheathed flagella resembling those of H. muridarum, H. rapproici and H. bilis. With the exception of H. muridarum, which has a helically coiled body, the other two are also straight to slightly curved fusiform bacteria.

It has been postulated that the spiral shape and the active flagella enable the Helicobacter species to move easily in a viscous environment such as the mucus of the gastrointestinal tract [17]. It is possible that the periplasmic fibres coiled around the length of the fusiform helicobacters may also be related to motility, as the ridges formed by them could increase torque in the mucus or the fibres themselves could be contractile and produce rotation of the cell [6, 16].

H. trogontum presented prominent intracellular granules of different shapes, as has been observed in other species of this genus [16, 18]. The nature of these granules differs in different prokaryotic organisms, but they almost always function in the storage of energy or serve as a reservoir of structural building blocks [19]. The positive metachromatic staining of the
Fig. 2. Ultra-thin sections of *H. trogontum* culture. **A,** detail of a tip of the bacterium showing the cell wall and the flattened end of the cell (bar = 0.1 µm); **B,** the 'polar membrane' (arrow) and granules (bar = 0.1 µm); **C,** two cells longitudinally sectioned showing the serrated appearance of the micro-organism contour given by the periplasmic fibres and the granular cytoplasm with irregular and spherical granules (bar = 0.5 µm); **D,** coccoidal form of a 4-day-old *H. trogontum* culture (bar = 0.25 µm).
H. trogontum granules suggests that they contain polyphosphate.

Although species that resemble H. trogontum, such as H. muridarum, H. rappini and H. bilis, are considered natural inhabitants of the intestine of rodents, they seem to have the ability to elicit disease when found at other anatomical sites of the host or in other host species. H. muridarum has occasionally been observed in the gastric mucosa of mice and rats where its presence is associated with gastritis [15, 16, 20]. H. bilis colonises, besides the lower intestine, the bile and liver of aged inbred mice and is associated with multifocal chronic hepatitis [8]. H. rappini is a normal inhabitant of the intestinal mucosa of mice, but has also been isolated from aborted sheep fetuses and stools of humans with chronic diarrhoea [14]. Another rodent species that is also associated with disease is H. hepaticus [7]. Although not sharing many morphological characteristics with H. trogontum, H. hepaticus is most closely related to it on the basis of 16S rRNA sequence analysis [11].

Investigations are required, to determine whether H. trogontum, like these other rodent species, is potentially virulent and, under certain circumstances, has the ability to colonise other anatomical regions of the gastrointestinal tract besides the colon in its natural host.

In gnotobiotic mice inoculated with H. trogontum, the bacterium could be detected in the small and large bowel. Another study with a larger number of animals is in progress in our laboratory to elucidate the pattern of H. trogontum colonisation and the histopathological changes it can induce in the gastrointestinal tract of gnotobiotic mice.

Several clusters of bacteria were seen inside the enterocytes, mainly in the caecum of the gnotobiotic animals. Tissue invasion by helicobacters has seldom been reported [21, 22]. H. pylori has been identified within the lamina propria of gastric epithelium, but mucosal invasion is rare [23]. Endocytosed bacteria in gastric mucosa were well documented in one ultrastructural study of H. mustelae in the ferret [24]. In a study by Phillips and Lee [25] on the pattern of colonisation of a spiral bacterium (lately classified as H. muridarum) in the intestine of conventional and gnotobiotic rodents, intracellular organisms were consistently seen only in the intestinal tissues of the latter. The authors have hypothesised that this phenomenon
Fig. 4. Electron micrography of the caecum of an infected mouse showing: A. *H. trogontum* within the lumen of a gland (bar = 1.0 µm); B, bacteria in clusters and free (arrowheads) in the cytoplasm of the epithelial cells (bar = 2.0 µm).
Fig. 5. Ultra-thin section of the caecum (A, B, C) and the colon (D) of a mouse infected with *H. trogontum* showing: A, a bacterial cluster occupying the entire cytoplasm and dislodging the nucleus to the top of the cell, note the loss of microvilli of the neighbouring cell (bar = 5.0 µm); B, degenerated cell without microvilli, presenting a pycnotic nucleus and structures with different shapes and densities (bar = 5.0 µm); C, damaged cell with several bacteria free (arrowhead) in cellular debris and structures with different densities (arrow) (bar = 2.0 µm); D, transverse section of a bacterium on the surface of a colon enterocyte (bar = 1.0 µm).
may occur either as a consequence of bacterial overgrowth leading to increased tissue invasion or as a consequence of the reduced defence mechanisms of gnotobiotic hosts. This could also be an explanation for the intracellular bacteria observed in the present study. However, the alterations in epithelial cells of the intestine, as well as the tissue invasion by the bacteria, observed in the present study seemed to be more pronounced than those reported by Phillips and Lee [25].

Intense vacuolation of the epithelial cells of the ileum were observed. Vacuolation has also been observed in cells of the antral mucosa of *H. pylori*-positive patients [26,27]. As a subset of *H. pylori* strains expresses a cytotoxin that induces cellular vacuolation in a number of different epithelial cell lines in vitro [28], it is important to investigate whether *H. trogontum* has the ability to produce a similar virulence factor. Other degenerative changes that have been observed in the gastric mucosa colonised by *H. pylori* [27], such as loss of microvilli and increased epithelial cell desquamation, were also observed in the large bowel of *H. trogontum*-positive mice.

These observations suggest that *H. trogontum*, although probably part of the indigenous microbiota of the colonic mucosa of rats, may have the ability to cause harm to the host under certain conditions, as demonstrated in the present study, where *H. trogontum* colonised the gastrointestina tract of a germ-free host.

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### References


Experimental Root Canal Infections in Conventional and Germ-Free Mice

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A small animal model was evaluated to study the interrelationships between microorganisms after their implantation in root canals (inferior central incisors) using germ-free (GF) and conventional (CV) mice. The selected microorganisms were: Porphyromonas endodontalis (ATCC 35406), Eubacterium lentum (ATCC 25559), Peptostreptococcus anaerobius (ATCC 27337), Fusobacterium nucleatum (ATCC 10953), Escherichia coli (ATCC 25922), and Enterococcus faecalis (ATCC 4083). Only P. anaerobius, E. coli, and E. faecalis, respectively, were able to colonize when inoculated alone into the root canal of both CV and GF mice. E. lentum, when inoculated alone colonized only in CV animals. P. endodontalis and F. nucleatum were unable to colonize in CV and GF animals after single inoculation. It is concluded that the experimental animal model presented herein is valuable for ecological studies of root canal infections and that only some strict anaerobic bacteria are able to colonize mice root canals when inoculated by themselves alone in pure culture.

Studying traumatized human teeth, with pulpal necrosis and a whole crown, Sundqvist (1) showed that microorganisms could only be isolated from patients who had developed a periapical lesion, confirming the interrelationship between periapical lesions and infection shown by Kakehashi et al. (2). Sundqvist (1) showed that 90% of the isolated bacterial species were anaerobes (1). Many other studies support these findings, demonstrating that mixed microbiota of root canals are made up mostly of anaerobes (3, 4).

Fabricius et al. (5), in an experiment with monkeys, inoculated indigenous oral bacteria in root canals after devitalization of the pulpal tissue and followed the development of the process up to 3 yr. Initially, there was a higher proportion of facultative anaerobes than of strict anaerobic bacteria. However, after 6 months, facultative bacteria were increasingly outnumbered and, when the observation period was longer, the predominance of the strict anaerobic bacteria became more pronounced. This experiment (as well as those reported by Tuni-Ishi et al. (6)) showed that, in the endodontic environment, some factors selected the sequential development of the anaerobic microbiota in specific proportions.

According to Sundqvist (7), there are many factors that could influence microbial growth and colonization in root canals. Some of these factors are abiotic, such as the availability of nutrients or the low oxygen tension in root canals with necrotic pulps. Others are biotic factors, such as the bacterial interactions that can be mutualistic (coordinated adhesion) or antagonistic (bacteriocin production).

In the present study, we developed a small size animal model that could be useful for the determination of the interrelationships between the ecological components of the root canal in eubiotic and dysbiotic conditions. For this purpose, we checked the ability of some bacteria prevalent in human endodontic infections to colonize experimentally the root canals of conventional (CV) and germ-free (GF) mice either in monosassociation or disassociation (i.e., when inoculated as one species only or inoculated jointly with another organism). A preliminary account of the results herein was reported (8).

MATERIALS AND METHODS

Animals

Groups of 2-month-old CV and GF NIH Swiss (Taconic, Germantown, OH) mice were used. The GF animals were kept in Trexler-type isolators (Class Biologically Clean, Madison WI). The CV mice were originally derived from the GF colony and kept in a CV animal room for many generations. For the experiments, GF animals were maintained in microisolators (UNO Roestvastuaal BV, Zevenaar, The Netherlands), and all the manipulations were conducted under aseptic conditions in a laminar flow hood (Veco, Campinas, SP, Brazil). All of the animals were fed an autoclavable commercial diet for rodents (Nuvital, Curitiba, PR, Brazil) ad libitum.
Bacteria

In accordance with the pertinent literature, the following bacteria were selected for inoculation: *Porphyromonas endodontalis* (ATCC 35406), *Peptostreptococcus anaerobius* (ATCC 27337), *Eubacterium lentum* (ATCC 25559), *Fusobacterium nucleatum* (ATCC 10953), *Escherichia coli* (ATCC 25922), and *Enterococcus faecalis* (ATCC 4083). The first four strict anaerobes microorganisms were maintained in an anaerobic chamber containing an atmosphere of: N₂, 85%; H₂, 10%; and CO₂, 5% (Forma Scientific, Inc., Marietta, OH). Oxygen sensitivity of the strict anaerobic bacteria has been checked. They were plated onto Petri dishes containing blood agar supplemented with hemin and menadione, withdrawn from the anaerobic chamber, and exposed to the atmospheric environment for 10, 30, 60, and 120 min, respectively. Then, they were returned to the anaerobic chamber for incubation at 37°C.

Root Canal Instrumentation

Before any surgical procedure, the animals were anesthetized intraperitoneally using diazepam (0.05 ml) as premedication and, after 5 min, sodium pentobarbital (35 mg/kg) as reported by Barbosa et al. (9). The inferior central incisors of the animal were isolated using an isolation kit consisting of a rubber dam (RD) frame and an orthodontic manufactured RD clamp. The teeth were opened using a low-speed handpiece and size one quarter sterile round burs. The root pulp was excised during the filing of the root canal using sterilized K-files from #15 to #30. Sterile saline was used as irrigating solution. All of the procedures were conducted in the laminar flow hood.

Radiographic Techniques

The radiographs were taken just before the instrumentation of the root canal. The anesthetized animals were immobilized on a wooden board lined with cork. The following radiographic criteria were selected: Focal object distance (FOD) = 10 cm, exposure time = ¼ s, radiographic angle = +75°. The radiographs were developed according to the manufacturer’s instructions.

Bacteria Inoculation

A 25 µl sample containing 10⁵ to 5 × 10⁴ viable cells of the selected microorganism was inoculated into the root canal through drilling, using type PPD syringes and needles. The purity of the sample was tested by Gram stain soon before its inoculation. The opening and inoculation in GF and CV animals were performed under absolute isolation in the laminar flow hood. Disinfection and antisepsis of the surgical field were performed according to Möller (3). All instruments were sterilized by autoclaving. All of the chemicals were of pure quality grade.

Analysis

Five days after inoculation, the teeth were opened and an absorbent cone was inserted. The paper cone was withdrawn from the root and smeared onto a plate containing blood agar supplemented with hemin and menadione that was incubated for 5 days at 37°C in the anaerobic chamber. After confirmation of microbial growth, a Gram stain was performed to check the cellular morphology of each inoculated sample.

Bacteriocin Production

The six selected bacterial strains were tested for their bacteriocinogenic activity. Each strain was tested for its inhibition activity against itself and also against the other five strains using the double layer method (10).

RESULTS

A total of 22 CV mice and 18 GF mice were used in the different experiments with pure cultures or some dissociations. Table 1 shows that only *P. anaerobius*, *E. coli*, and *E. faecalis* when in monoassociation were able to colonize the root canal of both CV and GF mice. In one CV animal, implantation of *E. faecalis* succeeded only after the second inoculation. In another CV animal, implantation was followed by the formation of a periapical abscess. The collection was made by aspiration and the content was inoculated in thioglycollate broth, thus confirming the noncontamination by Gram stain. *E. lentum* was implanted in monoassociation only in CV animals. On the other hand, *P. endodontalis* and *F. nucleatum* were unable to implant both in CV and GF animals. To check if a mutualistic effect through a disassociated inoculation could be helpful for the implantation of *P. endodontalis* and *F. nucleatum*, these microorganisms were inoculated together with a facultative (*E. faecalis* or *E. coli*) or a strict (*P. anaerobius*) anaerobic bacteria in GF mice. The same type of experiment was performed in GF mice using an *E. lentum + E. coli* inoculum. The mice inoculated with this latter bacterial dissociation presented a periapical abscess 5 days later. However, the results showed that only *E. coli* was implanted. The other experiments using a disassociated inoculum showed similar results when only the possible “helper” strain (*E. faecalis*, *E. coli*, or *P. anaerobius*) was implanted. A loss of viability of the *P. endodontalis* and *F. nucleatum* inoculum was not involved in this result, because the two strains remained viable in all stages of the experimental period, as shown by the oxygen sensitivity assays.

Table 2 shows the results obtained by the in vitro tests of the

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**Table 1. Experimental inoculation in root canals of CV and GF mice**

<table>
<thead>
<tr>
<th>Inoculated Bacteria</th>
<th>Mice Colonized/ Mice Inoculated</th>
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<tbody>
<tr>
<td></td>
<td>CV</td>
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<tr>
<td><em>Enterococcus faecalis</em></td>
<td>2/4</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>1/3</td>
</tr>
<tr>
<td><em>Peptostreptococcus anaerobius</em></td>
<td>1/3</td>
</tr>
<tr>
<td><em>Eubacterium lentum</em></td>
<td>1/4</td>
</tr>
<tr>
<td><em>Porphyromonas endodontalis</em></td>
<td>0/4</td>
</tr>
<tr>
<td><em>Fusobacterium nucleatum</em></td>
<td>0/4</td>
</tr>
<tr>
<td><em>E. lentum + E. coli</em></td>
<td>—</td>
</tr>
<tr>
<td><em>P. endodontalis + E. faecalis</em></td>
<td>—</td>
</tr>
<tr>
<td><em>E. nucleatum + E. coli</em></td>
<td>—</td>
</tr>
<tr>
<td><em>P. endodontalis + P. anaerobius</em></td>
<td>—</td>
</tr>
</tbody>
</table>

*Colonized only by *E. coli*.
†Colonized only by *E. faecalis*.
‡Colonized only by *P. anaerobius*.
bacteriocin-like activity of the selected strains. Two strains (*P.\nanaerobius* and *F.\nnucleatum*) showed both autoantagonism and some heteroantagonism. The other tested strains showed no anta-
gonism or only a relative heteroantagonism (*E.\ncoli* and *E.\nfaecalis*).

### DISCUSSION

Gnotobiotic animals are good models for the study of infectious
pathologies, because the synergistic or the antagonistic influences
of the resident microbiota or their byproducts may be detected.
These animal models are also very helpful in determining the real
effect and the identity of the etiological agent of an infectious
disease. GF mice and gnotobiotic rats were used to demonstrate the
involvement of bacteria in dental caries and periodontal disease
(11, 12). Rats, hamsters, dogs, and nonhuman primates are the
most frequently used animal models in dental disease research.
Mice are rarely used, and the literature concerning the dental
anatomy of this animal model is scarce. The use of X-ray, with a
radiological technique adapted for the experimental animal, al-
lowed the accurate distinction between root canal and periodontal
space. The root canal in mice is restricted to the apical 2 mm,
beginning below the gingival margin. The isolation kit used in this
work and made up by an RD frame and RD clamp from an
orthodontic manufacture was very effective.

Implantation of facultative anaerobic bacteria in mono-
infection occurred both in CV and GF mice (Table 1), which is in agreement with the data by Dahlén et al. (13) working with CV
monkeys. Specifically, the implantation of *E.\nfaecalis* in CV and GF mice, either in mono-inoculation or inoculated in association with an
anaerobic strain, was supported by previous studies (6). The oc-
ccurrence of acute periapical abscesses, elicited both by *E.\nfaecalis* and *E.\ncoli* in CV and GF mice, respectively, suggests the possi-
bility of a greater number of viable cells within the root canal (14)
due to the favorable conditions of the corresponding animals
allowing higher growth of these strains. It is relevant that the GF
animal, in which the abscess was observed, was inoculated with
both *E.\nlenum* and *E.\ncoli*, with no implantation of the former.
Sundqvist et al. (14) suggest that virulence may be related to an
increased ability of some microorganisms to resist phagocytosis or a
decrease of this function due to microbial action. In another
work, Sundqvist et al. (13) suggested that the mixed infections that
induce a purulent inflammation at the apical region would involve
a species of the strain *Bacteroides* spp. (4, 16). In the present
experiments, strains for this genus were not selected.

Previous data indicated that some strict anaerobic bacteria may
- be more powerful in inducing apical periodontitis. However, it has
- not been confirmed, up to the present, whether these bacterial
strains predominating in a mixed microbiota have a special ability
- to trigger an inflammatory process in monoinfection. Data shown

<table>
<thead>
<tr>
<th>Producing Strain</th>
<th>Target Strain</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
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<tbody>
<tr>
<td><em>Peptostreptococcus anaerobius</em> (A)</td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Eubacterium lentum</em> (B)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Fusobacterium nucleatum</em> (C)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
<td><em>Porphyromonas endodontalis</em> (D) NT*</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
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<tr>
<td><em>Escherichia coli</em> (E)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>NT</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em> (F)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>NT</td>
<td>-</td>
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<td>-</td>
</tr>
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</table>

*NT* = not tested.
This study demonstrates that the absence of an indigenous bacterial microbiota and the special anatomical conditions allowing a bacterial sampling free from contamination make the root canal, prepared as described herein, proper for the study of bacterial interactions and their relationships with the host.

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References


ANTAGONISM AGAINST VIBRIO CHOLERAE BY BACTERIAL DIFFUSIBLE COMPOUND IN THE FECAL MICROBIOTA OF RODENTS

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ABSTRACT

In an ex vivo agar plate assay, we monitored the appearance of an inhibitory halo against Vibrio cholerae from the feces of Wistar and Fischer rats aged 10 to 42 days. The frequency of Wistar rats showing halo increased from 0% (10 days) to a maximum of 80.0% (29 days) and then decreased to 53.3% (42 days). A similar pattern was obtained with Fischer rats but with a lower intensity (maximum frequency of 50.0% by day 36). In a separate experiment, when Wistar rats were fed a low-protein diet for 7 days, the inhibitory halo decreased drastically. Three apparently different colony morphologies were isolated from the dominant fecal microbiota: a facultative anaerobe (FAN) and two strict anaerobes (SAN). The ex vivo inhibitory test showed a halo around the feces of germfree mice monoassociated with the FAN bacterium or one of the SAN bacteria but not of the germfree ones. After oral challenge of all groups with V. cholerae, a permissive and a drastic barrier effects were observed in mice with FAN and SAN-associated bacteria, respectively. The FAN and one SAN bacteria used in the in vivo challenges were identified as Escherichia coli and Streptococcus intermedius, respectively. The potent antagonism developed by the rat intestinal microbiota against V. cholerae seems to be due, in part, to diffusible compounds and this phenomenon depends apparently on age, strain and nutrition of the animals. These preliminary results also suggest that this effect was due to more than one bacterial component at any given moment.

Key words: Vibrio cholerae, Escherichia coli, Streptococcus intermedius, antagonism, microbiota.

INTRODUCTION

The resistance of conventional rodents to intestinal colonization with human enteric pathogens such as Salmonella typhimurium, Shigella flexneri and Vibrio cholerae has been known for many years and the participation of the normal intestinal microbiota in this function was well demonstrated by experiments using rodents with an antibiotic-altered microbiota (4) and gnotobiotic animals (7). A study by Miller and Feeley (8) dealt with the inhibitory activity of some intestinal bacteria against V. cholerae. Germfree mice were associated with mixtures of V. cholerae and aerobic and anaerobic bacteria to determine which bacteria

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would eliminate V. cholerae from the mice. Not until a combination of Escherichia coli, Proteus mirabilis and Enterococcus faecalis was present in the intestinal tracts of the animals did V. cholerae disappear from the feces. However, the strains used for the mixture came from culture collections and different animal species, and some of them did not belong to the dominant intestinal microbiota in eubiosis.

The antagonistic phenomenon is potent but also fragile, being perturbed by factors such as drug ingestion, feeding and stress (12). The possible mechanisms of this bacterial interaction include competition for nutrients or for adhesion sites, stimulation of peristalsis and local immunity, and production of inhibitory substances (bacteriocins) or metabolites (fatty acids) (15).

This work describes the influence of age, sex, strain and low-protein nutrition on the production of a diffusible antagonistic substance against V. cholerae in the feces of rats. Isolation and partial identification of three bacteria producing these substances were also performed.

MATERIALS AND METHODS

Animals and Diets. Wistar and Fischer rats were obtained from the "Biotério do Instituto de Ciências Biológicas - UFMG, Belo Horizonte, MG", and "Departamento de Nutrição - UFOP, Ouro Preto, MG, Brasil", respectively. Two hundred rats of both sexes, and 10 and 15 or 22 days old were used at the beginning of the experiments on the influence of age, strain and sex or low-protein diet, respectively. To study the influence of age, strain and sex, the animals were fed a commercial diet for rodents (Nuvital, Curitiba, PR, Brasil). For the nutritional experiment, the low-protein diet used contained: casein, 20.0; corn starch, 951.5; corn oil, 80.0; cellulose, 10.0; choline, 2.4; vitamin E, 0.1; mineral mixture, 40.0 (6) and vitamin mixture, 10.0 (6). The same formulation was used for the control diet but with more casein (200 g) and corn starch adjusted (827.5 g) to obtain isocaloric control and hypoproteic diets.

—Germfree NIH mice (Taconic, Germantown, NY, USA) of both sexes were used as recipient animals for the screening of rat fecal bacteria responsible for the antagonistic compound production. Twenty-eight days old male Wistar rats fed a commercial rodent diet and whose fecal samples showed an inhibitory halo against V. cholerae were used as intestinal bacteria donors. The germfree animals were housed in flexible plastic isolators (Class Biologically Clean Ltd., Madison, WI.

Fig. 1. Frequency of an inhibitory halo against Vibrio cholerae caused by a diffusible substance from feces of 10, 15, 22, 29, 36 and 42 day old Wistar or Fischer rats fed a commercial diet for rodents.

"A Different letters indicate significant differences between the different ages for the same strain (P < 0.05).

b Different numbers indicate significant differences between the two strains for the same age (P < 0.05).

Fig. 2. Diameter of the inhibitory halo against Vibrio cholerae caused by a diffusible substance from feces of 10, 15, 22, 29, 36 and 42 day old Wistar or Fischer rats fed a commercial diet for rodents.

ac Different letters indicate significant differences between the different ages for the same strain (P < 0.05).
b Different numbers indicate significant differences between the two strains for the same age (P < 0.05).
Antagonism against *Vibrio cholerae*

USA), and handled according to established procedures (9). The animals received sterilized water in square pack bottle (American Sterilizer Company, Erie, PA, USA) and a commercial autoclavable diet for rodents (Nuvital, Curitiba, PR, Brasil) *ad libitum*. Experiments with gnotobiotic mice were carried out in microisolators (UNO Roestvasttuin B.V., Zevenaar, The Netherlands).

**Experimental Design.** To study the influence of age, strain and sex, we monitored the frequency of an inhibitory halo against *V. cholerae* from the feces of 100 Wistar or 100 Fischer rats of both sexes at the age of 10, 15, 22, 29, 36 and 42 days.

For the nutritional experiment, low-protein and control groups of 11 and 6 animals were used, respectively. The experimental diets were initiated for one week at the age of 22 days. After this time, both groups received the control diet for one more week. Inhibitory tests were performed at the age of 22, 29 and 36 days.

**Detection of an Inhibitory Diffusible Substance by in vitro and ex vivo Tests.** The *in vitro* assay for inhibitory diffusible substance was carried out by the double-layer method. The producing bacteria were spot inoculated on the surface of Brain Heart Infusion (BHI) agar or deMan, Rogosa and Sharpe (MRS) agar (Difco, Detroit, MI, USA) on a Petri dish. After incubation at 37°C for 48 h, the cells were killed by exposure to chloroform for 30 min. Residual chloroform was allowed to evaporate and the Petri dish was overlayed with 3.5 ml of BHI or MRS soft agar (0.7%) which had been inoculated with 0.2 ml of an 18 h culture of the indicator strain. After 24 h of incubation at 37°C, the dish was evaluated for the presence of an inhibitory halo.

For the *ex vivo* assay, freshly collected feces from rats (50 mg) or mice (10 mg) were placed on a Petri dish containing Thioglycollate-Bile Sucrose (TCBS) agar medium (Difco, Detroit, MI, USA) and incubated for 48 h at 4°C. The plates were then spread with 0.1 ml of a 10^1 dilution from a 24 h *V. cholerae* BHI broth culture and incubated for 24 h at 37°C. The presence of an inhibitory halo was observed around the feces and its diameter was measured. The *V. cholerae* sorogroup 01, biotype El Tor used in this study was isolated from a clinical case at the Fundação Ezequiel Dias (Belo Horizonte, MG, Brasil).

Screening of Rat Fecal Bacteria Producing an Inhibitory Compound Against *V. cholerae*. The dominant fecal microbiota of rats was obtained by decimal dilution under incubation in an anaerobic chamber containing an atmosphere of 85% N₂, 10% H₂, 5% CO₂ (Forma Scientific, Marietta, OH, USA) and plating 10^-1 dilution onto BHI agar. After incubation at 37°C for 7 days, three different colony morphologies were isolated and submitted to microscopic examination and respiratory and biochemical tests. Biochemical tests were carried out using the API 20 A, API 20 STREP and API 50 CH identification system kits (bioMérieux, Marcy-l’Etoile, France) for anaerobes, streptococci and carbohydrate metabolism of microorganisms, respectively. The BBL Crystal™ E/NF identification system kit (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) was used for enterobacteria.

**Association of Germfree Mice with Rat Fecal Bacteria.** The three bacterial isolates were grown separately on BHI agar for 48 h at 37°C in the anaerobic chamber, washed off with sterile buffered saline, and immediately associated with germfree mice. A 0.5 ml aliquot of these suspensions was used for inoculation of each animal through the oro gastric route. *Ex vivo* inhibitory tests were initiated 5 days after the germfree mice association with rat fecal bacteria. Challenge with *V. cholerae* was performed intragastrically with a 0.1 ml suspension containing about 10⁹ viable bacterial cells when an inhibitory halo was observed around the feces of the associated mice. Germfree mice challenged with the pathogenic bacteria were used as the control group.

**Vibrio cholerae** Counts. After oral challenge, freshly collected feces from gnotobiotic mice were submitted daily to decimal dilutions and counted on TCBS agar spread plates after 24 hr at 37°C.

**Statistical Analysis.** Inhibitory halo frequencies were compared by Fisher's exact test. Values of halo diameters between the different ages of the same rat strain were analyzed by 1-way analysis of variance. When a significant difference was found among groups (P < 0.05), a multiple comparison test was used to determine differences between groups. Values of halo diameters between the strain of rats for the same age were compared by the two-tailed Student t test. Results were considered to be significantly different only when P < 0.05. Statistical analysis were performed using an EPISTAT software package (T.L. Gustafson, Round Rock, TX, USA).

**RESULTS AND DISCUSSION**

Colonization resistance is the ability of the normal microbiota associated with humans and animals to protect these hosts against the unwanted establishment of pathogens (1). It is attributable to a complex interaction of many of the individual bacteria that comprise the mucosal microbiota. The production of antimicro-
bial substances is probably one of the most important mechanisms responsible for this antagonistic phenomenon (5). Bacteriocin-producing bacterial strains are commonly found in the mammalian digestive tract. While the presence of these antimicrobial metabolites has been demonstrated in vitro, it is unclear whether they are produced or have activity in vivo. Opposite data have been obtained in in vitro and in vivo experiments in which bacteriocin-sensitive Escherichia coli strains were found to inhibit bacteriocin-producing E. coli in the gnotobiotic mouse intestine (3). On the other hand, Ramaré et al. (11) showed the presence of an antibacterial substance in the feces of gnotobiotic rats monoassociated with a human Peptostreptococcus strain. This compound appeared to be produced through the concerted action of the host trypsin and a compound produced in vivo by the Peptostreptococcus. This last example shows the active participation of the host in a bacterial interaction occurring in the digestive ecosystem and demonstrates the importance of an in vivo model, such as gnotobiotic animals, to study these interrelationships.

Fig. 1 shows that the frequency of Wistar rats of both sexes showing an inhibitory halo against V. cholerae from their feces increased significantly ($P < 0.05$) from $0\%$ (10 days) to $80.0\%$ (29 days) and then decreased to $53.3\%$ (42 days). The values of halo diam-

![Fig. 3. Frequency of the inhibitory halo against V. cholerae caused by a diffusible substance from feces of 22 day old Wistar rats fed a control diet or a low-protein diet for one week and then nutritionally recovered with the control diet for the next week.](image)

Different letters indicate significant differences between the two diets ($P < 0.05$).

Fig. 4. Ex vivo inhibitory test against V. cholerae in TCBS cholera medium using feces from germfree mice (AX), transassociated mice with one facultative anaerobic bacterium and two strict anaerobic bacteria (3AN), monoassociated mice with the facultative anaerobic bacterium (1ANF) or dissociated mice with the two strict anaerobic bacteria (2ANS) from the rat fecal microbiota.

teter around these feces followed a similar pattern (Fig. 2), increasing significantly ($P < 0.05$) from $3.19 \pm 1.11$ cm (15 days) to a maximum of $4.72 \pm 1.24$ cm (29 days) and then decreasing to $2.03 \pm 0.58$ cm (42 days). In Fischer rats, the frequencies of animals showing an inhibitory halo against V. cholerae were lower (Fig. 1) throughout the experiment, but the pattern was similar when compared with Wistar rats (maximum frequency of $50.0\%$ by day 36). The halo diameter was also significantly smaller for Fischer rats (about 1 cm) when compared with their Wistar counterparts ($P < 0.05$), but relatively constant throughout the experiment (Fig. 2). Inhibitory halo frequency was similar in males and females throughout the experiment both in Wistar and Fischer rats (data not shown). Whereas the rat is naturally resistant to intestinal colonization by V. cholerae, the animals which did not show an inhibitory halo around their feces in the present study probably used a mechanism other than inhibitory diffusible compounds to eliminate the bacterial pathogen. The increase in inhibitory halo frequencies followed by a decrease observed in both Wistar and Fischer rats suggests a sequential colonization of the rat intestinal tract by different bacterial strains or a modification of the metabolic activity of the same bacterial strains as a function of age.
The gastrointestinal tract and its associated microbiota in human or animal beings constitute an open ecosystem with stable population and functional characteristics under normal environmental, physiological and nutritional conditions. Allogenic and autogenic factors can radically disturb this situation when the host is: (i) stressed in certain ways; (ii) starved or exposed to other forms of acute malnutrition; (iii) is given certain drugs, especially antibacterial compounds (2). It is well known, for example, that the normal microbiota of antibiotic-treated or stressed animals is markedly altered, and the breakdown of regulatory mechanisms in the gastrointestinal ecosystem allows easier establishment of pathogens in the tract (4,13).

The role of diet in modifications of the intestinal microbiota within the digestive ecosystem has been also the subject of numerous studies (10). In a separate experiment, when Wistar rats (22 days old and 63.64% frequency of inhibitory halo at the beginning of the experiment) were fed a low-protein diet for 7 days, the frequency of inhibitory haloes decreased significantly ($P < 0.05$) to 14% (Fig. 3). After nutritional recovery with the control diet for one week, this frequency returned to 50.0%.

Using the above data, we selected 28-day-old Wistar rats fed with a commercial diet for rodents and whose feces produced an inhibitory halo as fecal donor for the isolation of bacteria responsible for the antagonism against V. cholerae. From a 10^{-1} fecal dilution from this animal and after a 7 days incubation time at 37°C, we isolated 3 morphologically different colonies morphologies. The respiratory test showed that one of these bacteria was a facultative anaerobe (FAN) and the other two were strict anaerobes (SAN). These bacteria were associated with NIH germfree mice reared in microisolators. Five days later, the inhibitory test showed an antagonistic halo around the feces of the tricenic mice against the pathogenic bacterium (Fig. 4B). There was no halo from axenic animal feces (Fig. 4A). Then, two germfree mice groups were respectively monoassociated with the FAN bacterium and diassociated with the two SAN bacteria in separate microisolators. Large inhibition zones were observed for the two groups (Figs. 4C and D) but at different times (after 3 and 15 days of association for monoaxenic and dixenic animals, respectively). After oral challenge of monoassociated mice with V. cholerae, the bacterial pathogen was repressed (permisive barrier effect) to a population level of about 4.0 log CFU/g of feces in 3 days (Fig. 5). After the same oral challenge of diassociated mice, the bacterial pathogen was eliminated (drastic barrier effect) from the feces in about 2 weeks (Fig. 5). Then, germfree mice were associated for 5 days with one of the SAN bacteria when this microorganism reached fecal population levels of 9.6 log CFU/g. Smaller inhibition zones against V. cholerae were obtained from the feces of the monoassociated animals. Oral challenge of these mice with the bacterial pathogen resulted in its elimination from the mouse intestines in 5 days (Fig. 5). There was no inhibitory halo against the three pathogenic bacteria when an in vitro assay was carried out with them, independent of the medium used (BHI or MRS agar). This fact shows the limitation of the in vitro assay, because the production of inhibitory compounds against V. cholerae is apparently only possible in the host digestive ecosystem (as revealed by the ex vivo assay). Identification revealed Escherichia coli as the FAN strain. The two SAN bacteria were distinct Gram-positive cocci and the one used in the in vitro assay was identified as Streptococcus intermedius. The other SAN bacterium was an Extremely Oxygen Sensitive (EOS) strain. The presence in the rat gastrointestinal tract, at a given moment, of more than one antagonistic bacterial strain against V. cholerae showed that, as suggested by Freiter (2), the complex interactive system regulating the indigenous microbiota and its colonization resistance property involves redundancy, an important characteristic for the protection of the ecosystem.
The potent antagonism developed by the rat intestinal microbiota against *V. cholerae* seems to be due, in part, to diffusible compounds and this phenomenon depends apparently on the age, strain and nutrition of the animals. These preliminary results also suggest that the antagonistic effect of the rat intestinal microbiota against *V. cholerae* through diffusible substances, as observed in the *ex vivo* assay (but with different results from those obtained in the *in vivo* challenge, i.e., permissive and drastic barrier for the FAN and SAN bacteria, respectively), was exerted by more than one bacterial component at any given moment.

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RESUMO

O aparecimento de halo de inibição contra a *Vibrio cholerae* a partir das fezes de ratos Wistar e Fischer nas idades de 10 a 42 dias foi observado usando um teste *ex vivo* em placas. A frequência de ratos Wistar apresentando halo aumentou de 0% (10 dias) até um máximo de 80,0% (29 dias) antes de decair para 53,3% (42 dias). Um perfil similar foi obtido com os ratos Fischer mas com valores inferiores (frequência máxima de 50,0% no dia 36). Num experimento separado quando ratos Wistar foram alimentados com uma ração deficiente em proteína a frequência de halo diminuiu drasticamente. Três morfologias de colonias aparentemente distintas foram isoladas da microbiota fecal dominante: uma bactéria anaeróbica facultativa (ANF) e duas anaeróbias estritas (ANS). O teste inibitório *ex vivo* mostrou halo ao redor das fezes de camundongos isentos de germs monoassociados com a bactéria ANF ou uma das bactérias ANS mas não para os animais isentos de germs. Após desafio oral de todos os grupos com o *V. cholerae*, barreiras permissiva e drástica foram observadas em camundongos associados com as bactérias ANF e ANS, respectivamente. As bactérias ANF e uma ANS usadas em monoassociação nos desafos *in vivo* foram identificadas como *Escherichia coli* e *Streptococcus intermedius*, respectivamente. O potente antagonismo demonstrado pela microbiota intestinal de ratos contra *V. cholerae* parece devido, em parte, a compostos difusíveis e este fenômeno depende aparentemente da idade, de espécie e da nutrição do animal. Esses dados preliminares sugerem também que este antagonismo seja devido a mais de um componente bacteriano num instante dado.

Palavras-chave: *Vibrio cholerae*, *Escherichia coli*, *Streptococcus intermedius*, antagonismo, microbiota.

REFERENCES

Bacteriocin production by *Fusobacterium* isolates recovered from the oral cavity of human subjects with and without periodontal disease and of marmosets

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**SUMMARY**

Bacteriocin production has been studied in very few anaerobic bacteria, and no report is available for *Fusobacterium* species. In the present study a total of 167 *Fusobacterium* isolates were tested for bacteriocin production: 70 isolates were obtained from the oral cavity of patients with periodontal disease, 47 were recovered from healthy oral sites of human subjects and 50 from the oral cavity of *Callithrix penicillata*. Autoantagonism and isoantagonism were observed when the bacteriocin-producing isolates were tested against themselves. Heteroantagonism was detected by testing the *Fusobacterium* isolates against 14 reference strains and 2 strains of *Actinobacillus actinomycetemcomitans* from our laboratory collection. The auto-, iso- and heteroantagonism phenomena observed in this comparative study suggest a possible ecological role for this (these) antagonistic substance(s) in the oral environment.

Key-words: Antagonism, *Fusobacterium*, Bacteriocin, Mouth; Anaerobic bacteria, Antagonistic substances, Marmoset.

**INTRODUCTION**

The *Fusobacterium* genus, comprising anaerobic Gram-negative rod-shaped organisms, is found in the respiratory, genital and gastrointestinal tracts (Bennet and Eley, 1993; Jousimies-Somer et al., 1995). It is among the most frequently cultivable anaerobic bacteria isolated from human and animals, especially from oral sites (Edelstein, 1990; Jang and Hirsh, 1994).

Although fusobacteria are characteristically members of the indigenous microbiota, they have been implicated as opportunistic pathogens in several diseases, and *F. nucleatum* is the one most commonly isolated from clinical specimens (Bolstad et al., 1996). Fusobacteria are often associated with other aerobic, facultative and anaerobic bacteria in a synergistic relationship that can result in mixed infections such as periodontal disease (Karuna et al., 1995; Feuille et al., 1996).
Inhibitory substances may also be important factors in the interaction between indigenous and non-indigenous organisms. Several species of bacteria inhabiting the oral cavity produce antimicrobial substances called bacteriocins or bacteriocin-like substances (Hammond et al., 1987; Hohne et al., 1993; Novak et al., 1994), but there are no reports on bacteriocins produced by the genus *Fusobacterium*.

Similarities in pathogen susceptibility have made non-human primates ideal laboratory models, as they closely resemble humans in morphological, physiological and even social features, and are also eventual pathogen sources for them (Page, 1988; Ebersole and Kornman, 1991; Fantas et al., 1992; Miranda et al., 1996). During the 20th century, laboratory research on captive primates has elucidated the pathogenesis of many infectious diseases and facilitated drug and vaccine development. In the coming century, wild primate populations, an unexplored source of information regarding emerging infectious disease, may hold valuable clues to the origins and evolution of some important pathogens (Wolfe et al., 1998).

The aim of the present study was to determine the antimicrobial activity of *Fusobacterium* isolates obtained from human subjects with and without periodontal disease and from non-human primates (marmosets) in order to obtain comparative data. We also report the partial characterization of the antagonistic substance(s) produced by *Fusobacterium* spp.

**MATERIALS AND METHODS**

**Microorganisms**

A total of 167 isolates of *Fusobacterium* spp. were obtained from the oral cavity (subgingival plaques) of 10 human subjects with healthy periodontium (n = 47), 10 patients with periodontal disease (n = 70), and 10 *Caillitrest penticillata* (n = 50) reared in captivity, on a selective medium (Omata and Dugrady, 1956). Isolates were identified at the genus level by biochemical tests according to Holdeman et al. (1977) and Holt et al. (1994).


**Bacteriocin-like activity**

Bacteriocin-like activity was determined by the double layer method (Booth et al., 1977). The inoculum was prepared in brain-heart infusion broth supplemented with 0.5% yeast extract and 0.075% L-cystine (BHI-S) (Holdeman et al., 1977). Forty-eight-hour cultures were spotted simultaneously with a Steers' replicator onto the surface of BHI-S agar (1.5%) layered in 150 x 20 mm petri dishes and allowed to dry. After incubation at 37°C for 48 h in an anaerobic chamber (85% N₂, 5% CO₂, and 10% H₂) (Forma Scientific Co., USA), the cells were killed by exposure to chloroform for 30 min. Residual chloroform was allowed to evaporate and the petri dishes were overlaid with 6.0 ml of BHI-S soft agar (0.7%) which had been inoculated with 0.2 ml of a 24-h BHI-S broth culture of an indicator strain. A smaller inoculum (0.5 µl) of a 12-h culture in BHI-S broth was used for the *E. coli* indicator. After 24–48 h of incubation under anaerobiosis, the plates were evaluated for the presence of zones of growth inhibition and the halos were measured. Experiments were carried out in duplicate.

**Detection of interfering factors**

To rule out the possible presence of bacteriophages, a piece (3 mm in diameter) of agar at the inhibition zone was removed aseptically, according to the
method described by Turner and Jordan (98)). These blocks of agar were homogenized, centrifuged, placed on the surface of a BHI-S agar layered in 100×20 mm petri dishes and overlaid with 3.5 ml of BHI-S soft agar containing the indicator strain. After 48 h of incubation under anaerobiosis, the petri dishes were evaluated for the presence of lytic zones. This test was repeated at least twice. To search for possible inhibition by fatty acids, tests were conducted in BHI-S agar with and without the addition of 1% soluble starch (Walstad et al., 1974). The pH in the inhibition zone and in the non-inoculated medium was measured with a microelectrode (Microelectrode Inc., New Hampshire). The production of hydrogen peroxide was investigated by incorporating 0.03% (w/v) catalase (Sigma-1,000 U/ml) into the BHI-S agar while carrying out the antagonism test, as described by Hamada and Ooshima (1975). To exclude the possibility of residual chloroform interference in the growth of indicator strains, the evaluation of bacteriocinogenic activity was performed without its use. The producer isolates were not killed by exposure to chloroform and the indicator strains were inoculated with a loop near the spots of the producers, but without touching them (Farnas et al., 1992).

Partial characterization

The heat stability of the bacteriocin-like substance(s) was measured by submerging the agar plates, after growth of the producer cultures, to temperatures of 60, 70 and 80°C for 20 min before pouring the soft agar overlay. Sensitivity to several proteolytic enzymes was tested using buffered solutions as described by Gomori (1955) and Farnas et al. (1994). Fifty µl of enzyme solutions (protease K/Boehringer 50 µl/ml, type II trypsin Sigma 200 µl/ml, pronase/Cabiochem 1 mg/ml, all 0.01 M Tris-HCl buffer pH 7.8; pepsin/Sigma 200 µl/ml in 0.1 M citrate buffer pH 5.0; papain/Sigma 10 mg/ml in 0.1 M citrate phosphate buffer pH 7.0) were placed on the agar surface around the growing spots and allowed to cover areas larger than the expected zones of inhibition. Incubation was carried out for 3 h at 37°C. The effects of these enzymes were determined on the basis of their activity toward the inhibitory substances produced against E. lentum ATCC 25559. Two controls were run: one without the bacteriocin-producing isolate and with enzyme, and the other without enzyme and with the bacteriocin-producing isolate.

Statistical analysis

Data were analysed statistically by the two-tailed 𝜒² with Yate's correction, Fisher's exact test and Student's t test. The level of significance was set as 𝑝 < 0.05.

RESULTS

Table I shows the heteroantagonistic activity of 167 oral isolates of Fusobacterium spp. against 9 different indicator strains. The results indicate that 165/167 Fusobacterium isolates inhibited P. anaerobius ATCC 27337 and 117/117 inhibited the growth of E. lentum ATCC 25559, whereas 15/50 isolates from marmosets produced inhibitory substance(s) against B. fragilis ATCC 25586. The reference strain F. nucleatum ATCC 10953 also inhibited the growth of P. anaerobius ATCC 27337 and E. lentum ATCC 25559 but did not inhibit B. fragilis ATCC 25586. Only 31/70 and 23/70 Fusobacterium isolates obtained from patients with periodontal disease (table I, d) inhibited the growth of S. mutans IM/UFRI and S. sanguis ATCC 10557, respectively. With regard to S. sanguis ATCC 9927, 9167 Fusobacterium isolates were capable of inhibiting its growth. A. actinomycetemcomitans FDC Y4 was more frequently inhibited by Fusobacterium strains isolated from marmosets (40/50) than by isolates from patients (d) (29/70; 𝑝 < 10⁻⁴); strain FDC Y4 was not inhibited by isolates recovered from human subjects with healthy periodontium (table I, h). The strain P12 was more frequently inhibited by antagonistic substance(s) produced by isolates from marmosets (38/50) than by the ones isolated from human subjects (39/117; 𝑝 = 10⁻⁶). The A. actinomycetemcomitans P1421 growth was inhibited by 85/167 Fusobacterium isolates. When comparing isolates recovered from humans (h) to the ones obtained from patients (d), a significant difference in the activity of the antagonistic substance(s) produced was detected (𝑝 = 10⁻³).

None of the facultative bacteria tested (E. coli ATCC 25273, E. faecalis ATCC 4083 and S. aureus ATCC 25923) were inhibited either by the 117 Fusobacterium isolates tested or by F. nucleatum ATCC 10953. The black-pigmented anaerobes P. gingivalis FDC 581, P. intermedius ATCC 25611 and P. nigrescens ATCC 33562 were not inhibited by the Fusobacterium isolates tested: 31 from our collection and F. nucleatum ATCC 10953.

Table II shows the inhibitory activity of 53, 20 and 20 Fusobacterium isolates recovered from
Table 1. Bacteriocin(s) produced by 167 *Fusobacterium* spp. strains (heteroantigenism), isolated from the subgingival plaque of patients with and without periodontal disease and from marmosets (*Callithrix*).

<table>
<thead>
<tr>
<th>Producer</th>
<th><em>Fusobacterium</em> isolates recovered from:</th>
<th><em>Bacteroides</em></th>
<th><em>Streptococcus</em></th>
<th><em>Actinomyces</em></th>
<th><em>Peptostreptococcus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATCC 25586</td>
<td>ATCC 9927</td>
<td>ATCC 10557</td>
<td>ATCC 25559</td>
<td>ATCC 27337</td>
</tr>
<tr>
<td>marmosets</td>
<td>15/50 (*)</td>
<td>0/50</td>
<td>6/50</td>
<td>40/50</td>
<td>38/50</td>
</tr>
<tr>
<td>patients (d)</td>
<td>0/20</td>
<td>31/70</td>
<td>3/70</td>
<td>23/70</td>
<td>29/70</td>
</tr>
<tr>
<td>patients (h)</td>
<td>0/47</td>
<td>0/47</td>
<td>0/47</td>
<td>0/47</td>
<td>0/47</td>
</tr>
<tr>
<td>TOTAL</td>
<td>15/117</td>
<td>31/167</td>
<td>9/167</td>
<td>23/167</td>
<td>69/167</td>
</tr>
</tbody>
</table>

* d = with periodontal disease, h = with healthy periodontium; (*) bacteriocin producer isolates/total isolates.
Table II. Inhibitory activity of bacteriocin(s) produced by *Fusobacterium* isolates from the subgingival plaque of marmosets and of human subjects with and without periodontal disease.

<table>
<thead>
<tr>
<th>Producer × indicator</th>
<th>Isoantagonism</th>
<th>Antagonistic activity</th>
<th>Autoantagonism</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fusobacterium</em> isolates recovered from:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>patients (d) × patients (d)</td>
<td>43/53(*)</td>
<td>26/48</td>
<td></td>
</tr>
<tr>
<td>patients (h) × patients (h)</td>
<td>20/20</td>
<td>20/20</td>
<td></td>
</tr>
<tr>
<td>marmosets × marmosets</td>
<td>20/20</td>
<td>20/20</td>
<td></td>
</tr>
<tr>
<td>marmosets × patients (h)</td>
<td>16/16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>99/109</td>
<td></td>
<td>66/88</td>
</tr>
</tbody>
</table>

*d* = with periodontal disease; *h* = with healthy periodontum; (*) = bacteriocin producer isolates tested isolates; *p* = 0.007 when comparing the isoantagonism to the autoantagonism among patients with periodontal disease (d); *p* < 10⁻³ when comparing the autoantagonism among strains isolated from patients with periodontal disease to each of the other groups.

Patients (d), patients (h) and marmosets tested against themselves, and 16 marmoset isolates tested against 12 isolates recovered from patients (h). Isoantagonism (each isolate tested against the other ones) and autoantagonism (each one tested against itself) were observed, respectively, for 99/109 and 66/88 isolates. *F. nucleatum* ATCC 10953 showed isoantagonism against 50 out of 167 isolates, but not autoantagonism. With respect to autoantagonism, it was observed that it is less frequently detected among isolates from patients (d) than from human subjects (h) (*p* < 10⁻³) and from marmosets (*p* < 10⁻³). The results concerning expression of isoantagonism showed no statistically significant difference. It was observed that the occurrence of isoantagonism is more frequent than the expression of autoantagonism among patients with periodontal disease (d: *p* = 0.007). Figure 1 shows auto- and isoantagonism in an agar plate.

Testing for the presence of bacteriophages in the inhibition zone showed no lytic zones, indicating that the inhibition zones were not due to the presence of phages. The possibility of inhibition by fatty acids was ruled out because of the lack of interference upon addition of soluble starch to the medium. No significant variations in pH values in the inhibition zone and in the non-inoculated medium were detected. Production of hydrogen peroxide was not detected. The experiments carried out without the use of chloroform gave the same results as those that included exposure to chloroform to kill the producer isolates.
Inhibitory activity was eliminated by heating at 60, 70 and 80°C for 20 min. The inhibitory substance(s) produced by *Fusobacterium* spp. exhibited loss of activity after exposure to pronase, protease K and papain, but not after exposure to trypsin and pepsin.

**DISCUSSION**

The present study indicates that oral isolates of *Fusobacterium* produced bacteriocin-like antagonistic substance(s), showing auto-, iso- and heteroantagonism against a wide range of bacteria.

The selection of the indicator strains was based on their importance in the oral ecosystem. Some species, such as *S. uberis*, *E. coli*, *E. faecalis* and *S. aureus* were also included in the study to evaluate the spectrum of action of the antagonistic substance(s) produced. Also, some of them could eventually be transient organisms of the oral cavity, especially of marmosets.

The heteroantagonism observed is quite peculiar. It has been reported that the bacteriocins of Gram-negative bacteria are considered to have a more limited spectrum of action than those of Gram-positive bacteria (Reeves, 1965). The most studied bacteriocins from Gram-negative microorganisms, colicins, are toxic proteins produced by and active against *E. coli* and closely related bacteria (Pugsley, 1984). However, bacteriocins which act against taxonomically unrelated organisms have also been described (Tagg et al., 1976; Stevens et al., 1987; Hammons et al., 1987; Miranda et al., 1993; Farias et al., 1994; Miranda et al., 1996). The data reported here show that most *Fusobacterium* isolates tested inhibited more than one of the indicator strains and demonstrated activity against different genera of both Gram-positive and Gram-negative bacteria.

Regarding bacteriocins produced by oral Gram-negative bacteria other than *Fusobacteria*, we have very few studies on *A. actinomyctemcomitans* (Hammond et al., 1987; Stevens et al., 1987). *B. fragilis* (Farias et al., 1994) and black-pigmented anaerobes (Takada et al., 1991; Höfner et al., 1993). Hammond et al. (1987) reported that *A. actinomyctemcomitans* V4 produces a bacteriocin active against *S. sanguis*, *S. uberis*, *Actinomyces viscosus* and other *A. actinomyctemcomitans* strains, but not against other crevicular bacteria, including other streptococci and actinomyces. This bacteriocin was inhibited by exposure to heat for 30 min at 56°C, pronase and trypsin; this last enzyme could not inhibit the activity of the antagonistic substance(s) detected in our study. A strain of *B. fragilis* isolated from the oral cavity of *C. penicillata* was studied by Farias et al. (1994). The bacteriocin produced exhibited iso- and heteroantagonism and retained its activity after exposure to 60°C for 24 h. It was inhibited by pronase, trypsin, proteinase K and type VII protease. Regarding bacteriocins produced by black-pigmented Gram-negative anaerobes, Takada et al. (1991) showed that *Prevotella intermedia* strains isolated from subgingival plaques of patients with periodontitis can inhibit the growth of *P. intermedia* ATCC 25611 and *F. nucleatum*, but not of other crevicular bacteria, including *P. intermedia* ATCC 33563, *P. gingivalis*, *Capnocytophaga spuigena* and *A. actinomyctemcomitans*, as well as streptococci, actinomyces and veillonellas. The bacteriocin detected was inactivated by exposure to 90°C for 5 min, and protease. Höfner et al. (1993) studied the antimicrobial activity of strains of *P. intermedia* and *P. gingivalis* isolated from periodontal pockets against Gram-positive and Gram-negative anaerobes, as well as against each other. They report that *P. intermedia* presented higher antimicrobial activity than *P. gingivalis*, and only the first one was capable of inhibiting the growth of strains of the same species.

The Gram-positive organisms *S. sanguis* and *S. mutans* are members of the dominant indigenous microbiota of the oral cavity (Rogers, 1975). The characteristic alterations in the dental plaque microbiota, which are seen in the various forms of periodontal disease, indicate that species which are routinely found to compose a high proportion of healthy sites may decrease in number or even disappear in plaque specimens taken from sites of periodontal disease (Stevens et al., 1987). The Gram-negative periodontopathogen *A. actinomyctemcomitans* is related to localized juvenile periodontitis (Socransky and Haffajee, 1991). Hammond et al. (1987) reported that *A. actino-
mycoplasmatis FDC Y4 produces a bacteriocin active against S. sanguis but not against F. nucleatum. In our research, we found Fusobacterium isolates from patients (d) who exhibited inhibitory activity against S. mutans IM/UFRI, S. uberis ATCC 9927, S. sanguis ATCC 10557 and 3 strains of A. actinomycetemcomitans, including FDC Y4 (table 1). F. nucleatum is found in subgingival healthy sites in the oral cavity as well as in early stages of plaque-associated gingivitis and increases with the onset and progressive severity of periodontitis (Kornman, 1996). It is known that in some instances, F. nucleatum may account for as much as 90% of the crevicular microbiota in severe periodontitis (Rams et al., 1997). The indicator strains F. nucleatum ATCC 27337 and E. lentum ATCC 25589 showed higher sensitivity to bacteriocin-like substance(s) produced by Fusobacterium isolates (table 1). Pectostreptococcus spp. are commonly isolated from the subgingival plaque of healthy subjects and from patients with gingivitis, periodontitis and root infections. The Eubacterium genus is related to different abscesses, including those occurring in the oral cavity (Sunqvist, 1994). It appears that bacteriocin production would give an advantage to Fusobacterium for growth and persistence in oral ecological niches, especially in the colonization of periodontal sites in certain clinical conditions.

Bacteriocin-like substance(s) from marmoset Fusobacterium isolates had singular inhibitory activity on B. fragilis ATCC 25586, suggesting a possible difference in the bacterial relationships between the human and marmoset oral ecosystem. This fact may be related to coprophagy, an unusual practice among animals reared in captivity which may permit intestinal bacteria to persist in the oral cavity.

It might be expected that bacteriocinogenic strains are immune to the homologous bacteriocin. This is apparently due to synthesis of a specific protein which acts as an immunity factor (Tagg et al., 1976). However, autoantagonism is a well known phenomenon (Ryan et al., 1955; Yamada et al., 1987; Gilmore et al., 1990; Farias et al., 1992; Miranda et al., 1993) that seems to be more frequent in Gram-positive organisms (Jack et al., 1995). It has been found that the presence of the bacteriocin structural gene as well as the immunity gene are required for expression of immunity (Jack et al., 1995). It is possible that some Fusobacterium isolates do not encode for production of immunity proteins, what would lead to the sensitivity to their own bacteriocin. It is accepted that the "immunity breakdown" may occur owing to an imbalance in the concentrations of proteins that confer immunity to the cell itself (Levisohn et al., 1967). Immunity to bacteriocin is quite distinct from bacteriocin resistance which involves a loss of specific bacteriocin receptors on a previously sensitive cell (Turner and Jordan, 1981).

Competition among bacteria in the oral cavity can be achieved by factors such as metabolic byproducts, bacteriophages and bacteriocins (Tagg et al., 1976; Paul and Booth, 1988; Jack et al., 1995). The antagonism observed here was not due to chloroform action, bacteriophage, hydrogen peroxide, fatty acids or other acids produced by the microbial metabolism, as confirmed by the experiments. The antagonistic substance or substances detected are sensitive to high temperatures and exhibited loss of activity after exposure to some proteinases tested, what attests to their proteic nature.

Our results enable us to confirm that the inhibitory substance(s) detected and partially characterized is (are) a bacteriocin(s). So, this is the first report on bacteriocin production by Fusobacterium isolates.

Bacteriocins seem to contribute to the intraspecies and interspecies regulation of the microbiota (Jack et al., 1995). Although the natural role of bacteriocins has not been determined, they appear to be involved in microbial invasion and/or defense mechanisms (Tan and Riley, 1997). The auto- and heteroantagonism observed in the present study strongly suggest the ecological role of these substances in the oral ecosystem, since these phenomena influence the imbalance of specific microbiota at each site under normal and disease conditions. Our results reinforce previous findings regarding microbial interactions obtained by culturing oral specimens recovered from human subjects with periodontitis, includ-
ing a strong positive association between *Fusobacterium* spp. and *P. intermedius*, and a negative association between *Fusobacterium* spp. and *A. actinomycetemcomitans* (Rams et al., 1997). The spectrum of the bacteriocinogenic activity can express different pathogenic potential of indigenous bacteria.

Based on microbial associations in subgingival plaque samples, Socransky et al. (1988) pointed out that it seems likely that certain pathogens could colonize sites already colonized by some species more readily than others. Obviously, this may be as important in normal ecological conditions as in destructive diseases, but also, it may influence the success of therapy.

Thus, it seems clear that the antagonistic relationship that is critical for the stability of any microbial ecosystem could contribute to success or failure in colonization by putative pathogens.

In fact, studies on probiotics employing some experimental infectious disease animal models will probably lead to a better understanding of microbial associations in the oral ecosystem, which could be critical in providing protective species and long-term oral health by producing a stable, host-compatible oral microflora.

The bacteriocinogenic spectrum of the *Fusobacterium* isolates recovered from human subjects and marmosets included in his study was very broad. It is possible that each isolate may produce more than one antagonistic substance, with different physicochemical and biological properties. The differences in the inhibitory activities of isolates from human subjects with and without periodontal disease were remarkable. *Fusobacterium* isolates from diseased sites had a wider spectrum of activity than isolates from healthy human oral sites. This and further studies may be of taxonomic and epidemiological interest and might also contribute to the understanding of the role of these substances in oral microbial ecology of primates. For this purpose, purification and characterization of the substance(s) detected and others eventually produced by these microorganisms will be very important, and are currently under way in our laboratory.

Acknowledgements

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Production de bactériocines par des souches de *Fusobacterium* isolées de la cavité orale de sujets humains présentant ou non une maladie périodontale et à l'oiestrie

La production de bactériocine a été étudiée chez très peu de bactéries anabioles et aucune mention à ce respect n’est disponible pour les espèces du genre *Fusobacterium*. Dans cette étude, un total de 167 isolats du genre *Fusobacterium* a été testé pour la production de bactériocine: 70 souches ont été isolées de la cavité orale d’humains présentant une maladie périodontale; 47 souches ont été prélevées de sites oraux sains et 50 provenaient de la cavité orale de *Calitrichis punctilittu*. Des phénomènes d’auto- et d’isoantagonisme ont été observés lorsque les souches productrices de bactériocine(s) ont été testées entre elles. Un hétéroatantagonisme a été mis en évidence quand les isolats producteurs ont été testés contre 14 souches de référence et deux *Actinobacillus actinomycetemcomitans of the collection* de notre laboratoire. Les phénomènes d’auto- et d’iso- et d’hétéroatantagonisme suggèrent un possible rôle écologique de ces substances antagonistes dans l’environnement oral.

*Mot-clés: Antagonisme, Fusobacterium, Bactériocine, Bactéries anabioles, Bouche: Substances antagonistes, Oiestrie.*

References


BACTERICIDON OF ORAL FUSOBACTERIUM


Dose effect of oral *Saccharomyces boulardii* treatments on morbidity and mortality in immunosuppressed mice

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Survival, weight loss, translocation and histological alterations in the terminal ileum, liver and spleen were studied in mice simultaneously immunosuppressed with cyclophosphamide and treated or not with *Saccharomyces boulardii* until the death of all animals. The animals were divided into five groups: C1 (not immunosuppressed, not treated); C2 (immunosuppressed, not treated); B1 (immunosuppressed, treated with *S. boulardii* 10.0 mg); B2 (immunosuppressed, treated with *S. boulardii* 1.0 mg) and B3 (immunosuppressed, treated with *S. boulardii* 0.1 mg). Survival was higher in group B3 than in the other immunosuppressed groups. Weight loss was observed for all groups except C1. By day 7, some animals from each group were killed by ether inhalation for the determination of bacterial translocation and histopathological examination. Bacterial translocation to the liver was lower in groups C1 and B3 than in the other groups. The highest translocation to the liver and spleen was observed in group B1. Low *S. boulardii* translocation was observed in some animals, principally to the mesenteric lymph nodes. Histopathological examination showed a decrease in epithelial cell turnover with villus length reduction and loss of brush borders in group C2. Relative protection against these alterations was obtained when the animals were treated with the yeast, independently of the dose. Higher expression of the lymphoid component was also noted in the ileal lamina propria. Liver and spleen of mice treated with the yeast, together with activation of the reticulo-endothelial system, when compared with group C2 where lymphocyte depletion was observed. This study suggests a relative protection of immunosuppressed animals by treatment with *S. boulardii*, but this phenomenon was inversely proportional to the yeast dose.

**Introduction**

Aggressive cytostatic treatment applied for remission induction therapy in leukemic patients results in increasing granulocytopenic episodes, as well as serious damage to the epithelial and mucosal barriers and deterioration of humoral defence and cell immunity. Therefore, severe bacterial and deep mycotic infections are the main cause of complications and death in patients with acute leukemia [1]. The majority of these severe infections are of endogenous origin, i.e., caused by micro-organisms that have colonised the mucosal surface of the gastrointestinal (GI) tract and then have translocated.

Translocation is defined as the passage of viable microbes from the GI tract across the mucosal barrier to extra-intestinal sites [2]. Bacterial translocation, as measured by the appearance of viable bacteria in extra-intestinal organs, is promoted by three major mechanisms: (i) physical disruption of the mucosal barrier [3], such as occurs with ischaemia and reperfusion injury during endotoxic or haemorrhagic shock; (ii) intestinal bacterial overgrowth following disruption of the GI ecology by oral antibiotics, protein malnutrition or shock; and (iii) decreased host immune defences [4], resulting from immunosuppressive drugs or diseases such as cancer and AIDS.

The growing number of immunological incompetence situations promoting translocation has justified research on immunotherapeutic agents which may
potentiate the resistance of an incompetent host. Biotherapeutic agents (probiotics) are known to offer an alternative to conventional antimicrobial agents to which many pathogenic microorganisms eventually develop resistance [5]. A study in healthy human volunteers showed that oral administration of *Saccharomyces boulardii* activates both the complement and reticulo-endothelial systems [6]. No serious adverse effects have been reported with the biotherapeutic agents tested to date [5]. An obvious concern in the use of biotherapeutic agents is administration to severely immunocompromised or debilitated patients such that invasion into the systemic compartment might occur. However, there is limited information on the use of biotherapeutic agents in immunocompromised hosts. In mice immunosuppressed with prednisolone, orally administered *S. boulardii* decreased both the incidence of *Candida albicans* translocation to the mesenteric lymph nodes (MLN), liver and kidneys, and the number of translocating *C. albicans* per gram of MLN, spleen and kidneys [7]. *S. boulardii* was used in a double-blind, placebo-controlled trial in patients infected with HIV and presenting with chronic diarrhea; 56% of the patients treated with the yeast had resolution of this diarrhea compared with 6% of the patients on placebo [8]. Beyond the initial clinical trials of effectiveness against various diseases, biotherapeutic agents need to be characterised with the same dose-ranging studies expected for standard chemical investigational drugs. The published studies have rarely presented data on dose-dependent efficacy of the agents.

This study examined the effect of different oral doses of *S. boulardii* on survival, microbial translocation and histological alterations in the terminal ileum, liver and spleen of mice immunosuppressed with cyclophosphamide.

**Materials and methods**

**Mice**

Male NMRI Swiss mice (Central Institute for Animal Breeding, Hannover, Germany), 1 month old, were used (initial weight 20.04 SD 1.36 g). The animals were given a commercial diet (Nuvital, Curitiba PR, Brazil) for rodents and water *ad libitum*.

**Experimental design**

The animals were divided into five groups: C1 – not immunosuppressed, not treated; C2 – immunosuppressed, not treated; B1 – immunosuppressed, treated with 10.0 mg of *S. boulardii*; B2 – immunosuppressed, treated with 1.0 mg of *S. boulardii*; and B3 – immunosuppressed, treated with 0.1 mg of *S. boulardii*. The mice were weighed at the beginning of the experiment and at day 7, when some of the animals from each group were killed by ether inhalation to determine translocation and for histopathological examination. The cumulative mortality was observed in the remaining mice until the end of the experiment, at day 30.

**Treatments**

In the immunosuppressed model, mice were given cyclophosphamide (Enduxan; Shionogi and Co. Ltd, Osaka, Japan) 100 mg/kg by intraperitoneal injection on alternate days. When mice were given *S. boulardii* (B groups), the treatment was given concurrently by daily intragastric intubation with 0.1 ml of yeast suspension during the same period. Lyophilised *S. boulardii* (Floratil; Merck SA, Rio de Janeiro, Brazil) were suspended to the appropriate concentration in sterile PBS. In control animals (C groups), the same injection schedule was followed with saline for injection or intubation, or both.

**Microbial translocation test**

The MLN, liver and spleen of each mouse killed after days were removed aseptically, weighed and homogenised in a sterile grinding mortar. Ten-fold dilutions of the samples were performed and used for bacterial and yeast counts by plating on to Brain Heart Infusion Agar (Difco) and Sabouraud Dextrose Agar (Difco), respectively. Plates were incubated overnight or for 72 h at 37°C before bacterial and yeast counts, respectively. Bacterial translocation was considered to be positive for values = 10^2 CFU/g of tissue for liver and spleen and 10^3 CFU/g of tissue for MLN. For *S. boulardii*, translocation, the presence of any number of colonies was considered positive.

**Histopathological examination**

Fragments of the terminal ileum, liver and spleen were fixed with formaldehyde 4% and processed for paraffin embedding, and histopathological sections (3–5 μm) were stained with haematoxylin-eosin. The slides were all examined by the same pathologist, who was unaware of the experimental conditions of each group. The slides were coded and decoded only after the report had been written.

**Statistical analysis**

The Kaplan–Meier method and Wilcoxon and log-rank tests were used to compare survival times. Differences in translocation proportions were assessed by Fisher’s exact test. Comparisons of weight gain or loss were made by analysis of variance and co-variance and the Duncan multiple comparison method. Statistical analyses were performed with the Statistical Analysis System software (SAS Institute, Cary, NC, USA).
Results

Survival

Fig. 1 shows the life-table survival curves (Kaplan-Meier method) for the different experimental groups and the C2 control group. Survival was significantly higher (p = 0.0255) in group B3 (0.1 mg of S. boulardii) when compared with the other immunosuppressed groups (Wilcoxon test). There was no mortality in the C1 control group.

Weight variation

During the experiment, a weight loss was observed in all immunosuppressed groups (−4.03, −2.39, −3.40, and −3.81 g for C2, B1, B2 and B3, respectively) and a weight gain in the C1 control group (+2.79 g).

Fig. 1. Life-table survival curves (Kaplan-Meier method) for immunosuppressed, untreated mice (C2, ---), immunosuppressed mice treated with 10.0 mg of S. boulardii (B1, ---), immunosuppressed mice treated with 1.0 mg of S. boulardii (B2, ~ ---~) and immunosuppressed mice treated with 0.1 mg of S. boulardii (B3, --- ~). Despite the progressively higher weight loss with a decreased yeast dose, there was no significant difference between these values for the immunosuppressed groups. Analysis of co-variance with weighted least squares showed a statistically significant difference only between the two control groups C1 and C2 (p < 0.05).

Translocation

Table 1 shows the bacterial translocation index to MLN, liver and spleen for each group tested after treatment for 7 days. Statistical comparison was performed by the Fishers exact test between individual data of each group and for the same organ. The bacterial translocation index to the MLN was statistically similar for all groups. Translocation to the liver was lower in groups C1 and B3 when compared to the other groups (p = 0.0021). The greatest translocation to the liver was observed in group B1 (10.0 mg of S. boulardii). Higher bacterial translocation was also observed in the spleen of group B1 animals, but in this case the results with the C2, B2 and B3 groups were statistically similar (p = 0.00015). When statistically similar data were grouped (C1-B3; C2-B2; B1 for liver and C1; C2-B2-B3; B1 for spleen) and these groups were compared again by Fisher's exact test, the results obtained above for individual animals were confirmed and reinforced (p = 0.00033) for grouped liver data and p = 0.000011 for grouped spleen data). Translocation of S. boulardii was observed principally to the MLN. Translocation of the yeast to liver and spleen occurred rarely and always in animals with simultaneous significant bacterial translocation. In this case, the translocation levels of S. boulardii never exceeded 2.0 or 1.0 log10 cfu/g of organ for MLN or liver and spleen, respectively. There was no translocation of yeasts of the normal GI microbiota in the C2 group (data not shown).

Histopathology

Histopathological examination of the terminal ileal mucosa showed a decrease in epithelial cell turnover, flattened enterocytes and brush border discontinuity in

<table>
<thead>
<tr>
<th>Group</th>
<th>MLN</th>
<th>Liver</th>
<th>Spleen</th>
<th>Number of mice tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>2 (14.3)</td>
<td>3 (21.4)</td>
<td>0 (0.0)</td>
<td>14</td>
</tr>
<tr>
<td>C2</td>
<td>5 (22.7)</td>
<td>14 (63.6)</td>
<td>12 (54.6)</td>
<td>12</td>
</tr>
<tr>
<td>B1</td>
<td>6 (50.0)</td>
<td>1 (91.7)</td>
<td>10 (83.3)</td>
<td>12</td>
</tr>
<tr>
<td>B2</td>
<td>2 (16.7)</td>
<td>6 (50.0)</td>
<td>6 (50.0)</td>
<td>12</td>
</tr>
<tr>
<td>B3</td>
<td>3 (20.0)</td>
<td>5 (33.3)</td>
<td>6 (40.0)</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>0.269</td>
<td>0.0021</td>
<td>0.00015</td>
<td></td>
</tr>
</tbody>
</table>

*Non-immunosuppressed, non-treated mice; C2, immunosuppressed, untreated mice; B1, immunosuppressed mice treated with 10.0 mg of S. boulardii; B2, immunosuppressed mice treated with 1.0 mg of S. boulardii; and B3, immunosuppressed mice treated with 0.1 mg of S. boulardii.

*Significantly different, p < 0.05.

*In each group, comparison of individual data for the same organ by the Fisher's exact test.
group C2 (Fig. 2A). Relative protection against these alterations was obtained when the animals were treated with the yeast, independently of the dose (Fig. 2B). Higher expression of the lymphoid component in the ileal lamina propria, liver and spleen was also noted in mice treated with the yeast, including an activation of the reticulo-endothelial system, when compared with group C2 where lymphocyte depletion was observed (Fig. 2A and B, Fig. 3A and B).

Discussion

The GI tract has been demonstrated to be a reservoir for bacteria causing life-threatening infections in cancer patients undergoing chemotherapy [9], in bone marrow recipients [10] and in patients with AIDS [11]. Bacterial translocation from the GI tract is an important early step in the pathogenesis of opportunistic infections for these debilitated patients.

It seems that all the components of the host immune system - including mucosal, cell-mediated and humoral immunities - are involved in reducing bacterial translocation. Secretory immunoglobulin A (sIgA) is thought to inhibit the close association of bacteria with the mucosal epithelium, and so to reduce bacterial penetration. Cell-mediated immunity in the lamina propria and in lymphoid organs provides the second line of host defence against bacterial translocation. The complete depletion of either CD4+ CD8+ T cells, or both, in the intestinal epithelium lamina propria and MLN promoted bacterial translocation [12]. Serum immunoglobulins probably act as opsonins to increase the effectiveness of phagocytosis by macrophages and polymorphonuclear leucocytes and subsequent killing of translocating bacteria. In the case of a successful translocation, the reticulo-endothelial system (RES) plays a key role in removing micro-organisms from the circulation [13], and hepatic phagocytosis by Kupffer cells accounts for > 80% of RES function [14].

In this era of AIDS and selective immunosuppressive therapies which promote translocation, there is substantial interest in developing new methods of immunostimulation such as the use of probiotics. However, before a new substance can be used as an immunomodulator, it will have to be checked, not only regarding its efficiency in the enhancement of the immune response, but also as regards the absence of adverse side-effects for the host.

The results of the present study suggest that at a low
dose (0.1 mg) S. boulardii can reduce bacterial translocation from the GI tract (Table 1) and consequently the mortality (Fig. 1) of mice immunosuppressed with cyclophosphamide. It is not known precisely how oral S. boulardii treatment protects the host against infections by either their indigenous GI microbiota or by recently acquired exogenous microbes, although several properties of the yeast may contribute to its efficacy. For example, S. boulardii is reported to antagonize populations of certain microbial pathogens such as A. c事项 [15], but not of Salmonella typhimurium or Shigella flexneri [16]. On the other hand, another important effect of the yeast treatment is the stimulation of non-specific antiinfectious defenses such as the complement system and the phagocytic mononuclear system [6]. Glucan and other Saccharomyces extracts are important RES stimulators. The effects of these yeast products have been partially clarified. Proliferation of pluripotent haematopoietic stem cells, granulocyte-macrophage colony-forming cells and erythroid colony-forming cells in murine bone marrow has been demonstrated after glucan administration [17, 18]. Furthermore, after glucan administration, there is an increased production of interleukin 1 either by a direct effect on macrophages or indirectly by an increase in colony stimulating factor(s) production from glucan-stimulated T-cells [20]. The protective effect obtained with oral S. boulardii treatment and the simultaneous activation of the reticuloendothelial system was confirmed histopathologically. The higher expression of lymphoid components in the ileal lamina propia, liver and spleen of mice treated with the yeast was also observed in previous works [16, 17]. A significant increase in Escherichia coli blood clearance, as an indicator of the host's mononuclear phagocyte system activity, was found in gnotobiotic mice mono-associated with S. boulardii when compared with germfree animals [22].

In group C1 mice, a low "physiological" bacterial translocation was noted similar to the rates reported by others [2]. As suggested by Berg [23], this very low level of translocation of indigenous bacteria could be beneficial in priming the host immune response to improve defense against overt or opportunistic pathogens.

Consequently, although the results presented herein are only preliminary, the experiments were conducted on an animal model, the reduction of bacterial translocation and mortality by a low oral dose of S. boulardii suggests a possible therapeutic benefit of the probiotic for patients at risk for opportunistic infections by their indigenous GI microbiota.

References


PROBIOTICS STIMULATE THE MONONUCLEAR PHAGOCYTE SYSTEM OF MICE


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The influence of three probiotics (Lactobacillus acidophilus NCFM, Escherichia coli EMO, Saccharomyces boulardii) on the host mononuclear phagocytic system (MPS) was investigated through an in vivo E. coli B51 clearance test in gnotobiotic and conventional NMRI mice. Conventional mice were immunosuppressed with cyclophosphamide treatment at the time of probiotic treatment. Monoassociation with the respective probiotics and immunosuppression with the drug were performed for seven days before the experiments. A significant decrease in the clearance capacity was observed both in germfree and conventional immunosuppressed animals when compared with the conventional control group. An increase of host phagocytic system activity was observed in all monoxenic groups with only a small difference in extent and kinetics of the clearance. Treatment with L. acidophilus NCFM restored the clearance capacity in conventional immunosuppressed animals. S. boulardii improved the clearance capacity, but there was no statistical difference between data of the control and treated group. There was no improvement in clearance of immunosuppressed animals treated with E. coli EMO. These results demonstrate the important role of the intestinal microbiota and probiotics in host defense mechanisms involving MPS.

INTRODUCTION

The search for probiotics—preparations of live microorganisms that have some beneficial influence on maintenance of balanced intestinal microflora and improvement of the host's immune system—has gained significant impulse in the last few years. Previous studies have established an important role for probiotics such as strains of Bifidobacterium, Escherichia coli 083, E. coli EMO, Lactobacillus acidophilus, Lactobacillus casei and Saccharomyces boulardii in stimulating resistance against various infectious agents. This inhibitory action of probiotics may result from competition for nutrients and adhesion sites or by production of specific antimicrobial compounds. Recent studies have shown that probiotics may also be involved in stimulation of the immune system. Both L. acidophilus and Streptococcus thermophilus have been shown to significantly enhance the enzymatic and phagocytic activities of peritoneal macrophages. However, evidence supporting direct influence of probiotics on the host's phagocytic system is still poor. Although controlled laboratory testing of probiotics may shed some light on their effects and mechanism of action, the use of conventionally reared experimental animals as models is impaired by the activity of their normal flora, and results cannot be extrapolated to humans. In contrast, the use of germfree animals as models allows for the study of the sole effect of probiotics or in association with prepara-

cytic activities of peritoneal macrophages. In this study we tested the influence of selected probiotics (L. acidophilus NCFM, S. boulardii and E. coli EMO) on the capacity of germfree and cyclophosphamide-treated mice to clear an intravenously injected pathogen. We found that both germfree and immunosuppressed hosts are less capable of clearing pathogenic E. coli introduced systemically. Moreover, we found that all pro-

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Table 1: Probiotics population in feces of gnotobiotic mice during monoassociation

<table>
<thead>
<tr>
<th>Probiotic</th>
<th>Days after association</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>L. acidophilus NCFM</td>
<td>1.8 x 10^8</td>
</tr>
<tr>
<td>S. bouardii</td>
<td>nd</td>
</tr>
<tr>
<td>E. coli EMO</td>
<td>nd</td>
</tr>
</tbody>
</table>

1CFU/g of feces (data is average of five mice).
2The feces of these animals were plated in MRS agar and incubated at 37°C for 48 hours.
3The feces of these animals were plated in Sabouraud agar and incubated at 37°C for 72 hours.
4The feces of these animals were plated in MacConkey agar and incubated at 37°C for 24 hours.
nd = not done.

Probiotics could restore, at least partially, the capacity of germfree mice to clear bacteria from their MPS. In contrast, this was possible only by treatment with L. acidophilus NCFM in immunosuppressed hosts.

MATERIALS AND METHODS

Animals

Germfree NMRI Swiss mice were originally purchased from the Zentral Institut für Versuchstierzucht (Hannover, Germany) and used as matrices to derive the conventional colony. Both the conventional and germfree colonies had been kept in the Gnotobiology Laboratory of the Department of Biochemistry and Immunology of the Universidade Federal de Minas Gerais, Brazil, for two years in Trexler-type PVC isolators (Class Biologically Clean, Madison, WI). Microbiological control of germfree status of the-colony was performed as described by Pleasants12.

Microbial strains

Probiotics used in this study were: Lactobacillus acidophilus NCFM (Oklahoma University strain, kindly supplied by the University of Viçosa, MG, Brazil), Saccharomyces bouardii (kindly provided by Merck SA, São Paulo, Brazil) and Escherichia coli EMO (collection of the Department of Microbiology, UFMG, Belo Horizonte, Minas Gerais, Brazil). The pathogenic bacterium E. coli B41 (Department of Microbiology, UFMG) was used as the phagocytic test object in all clearance experiments.

Monaassociation with probiotics

Germfree animals were monoaasociated with probiotics per os. A single dose containing 10^8 L. acidophilus or E. coli EMO, or 10 mg of S. bouardii, in 0.1 ml of saline was given, under axenic conditions. Minoaasociated mice were subsequently maintained in sterile microisolators (UNO Roestvastaal BV, Zevenaar, The Netherlands). The success of the monoaassociations was checked by investigating the number of microorganisms per gram of feces. The association was also demonstrated by determination of the number of bacteria in several parts of the gastrointestinal tract using the same methodology for determination of bacterial counts in feces.

Table 2: Probiotic population along the gastrointestinal tract of monoaasociated hosts after seven days of monoaassociation

<table>
<thead>
<tr>
<th>Probiotic</th>
<th>Stomach</th>
<th>Ileum</th>
<th>Cecum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wall^b</td>
<td>content^c</td>
<td>wall^b</td>
</tr>
<tr>
<td>L. acidophilus NCFM</td>
<td>3.9 x 10^8</td>
<td>3.7 x 10^5</td>
<td>1.3 x 10^7</td>
</tr>
<tr>
<td>S. bouardii</td>
<td>7.8 x 10^4</td>
<td>8.7 x 10^5</td>
<td>2.1 x 10^7</td>
</tr>
<tr>
<td>E. coli EMO</td>
<td>8.7 x 10^4</td>
<td>0</td>
<td>4.4 x 10^5</td>
</tr>
</tbody>
</table>

^1CFU/g (data is average of five mice).
^2Part of the organ was taken, weighted, and plated in different media according to the microorganism analyzed.
^3Part of the content of the organ was taken, weighted, and plated in different media according to the microorganism analyzed.
**Fig. 1:** Clearance of *E. coli* B₄₁ in germfree (circles), conventional (squares) and cyclophosphamide-treated (triangles) conventional mice.

*E. coli* was injected at time 0 and mice were bled from the retroorbital plexus immediately or at 15, 30, 60 and 90 minutes. Results are expressed as the percentage of colonies found in relation to time 0 per ml of blood. Each point represents the average of five mice. Vertical bars stand for standard deviations of the means. Cyclophosphamide treatment was given as described in Materials and Methods. Data are from one representative experiment out of two performed. * indicate statistically significant difference (p<0.05).

**Immunosuppression**

Immunosuppression was induced by treating mice with four doses of cyclophosphamide (100 mg/kg body weight) given i.p. every 48 hours over seven days. Probiotics were associated daily at the same time of cyclophosphamide treatment as described above.

**Clearance assay**

Clearance assays were performed on day seven of monoassociation. The technique was performed as described previously. Briefly, an 18 hour culture of *E. coli* B₄₁ containing approximately $10^6$ bacteria/ml was centrifuged (2000 x g) and resuspended in sterile saline at $10^5$ bacteria/ml. Numbers of bacteria were determined by turbidity ($\lambda$ = 550nm) and optical density was adjusted up to 50. Each mouse was injected with 0.2 ml of this suspension in the tail vein. Blood samples were collected from the retroorbital plexus immediately after injection of *E. coli* B₄₁ and at 15, 30, 60 and 90 minutes after injection. Ten-fold serial dilutions of blood samples were cultured onto Petri dishes containing MacConkey medium. Dishes were incubated overnight at 37°C and colonies counted. Results are expressed as percent colony forming units of the counts obtained for time 0. Means were compared by Student's $t$ test.

**RESULTS**

The monoassociations were successful (Table 1). The microorganisms remained at high counts in feces (up to $10^7$ CFU/g). *E. coli* EMO reached the highest numbers in feces but was inhibited in the stomach (Table 2) where *L. acidophilus* NCFM was more resistant.

We compared the dynamics of different groups of animals on clearing the pathogen *E. coli* B₄₁ from systemic circulation. For this,
2 x 10^7 bacteria were injected intravenously into the tail vein and blood samples were collected and cultured several times after injection. We found that germfree animals had an impaired clearance capacity when compared to their conventional counterparts (Fig. 1). Thus, while in 90 minutes conventional animals had reduced the number of bacteria by a factor of five logs, germfree mice brought the number of bacteria down only one logarithmic unit. Interestingly, cyclophosphamide-treated conventional mice showed similar clearance capacity to that of germfree animals, and could not clear bacteria from circulation as efficiently as the conventional controls (Fig. 1).

The ability of the selected probiotics to restore the clearance capacity in germfree animals was investigated. Germfree mice were associated for one week with each probiotic, and the clearance assay was performed. As can be seen in Fig. 2, associations with all probiotics improved the capacity of germfree mice to clear E. coli B41 injected systemically. Thus, for up to 60 minutes, L. acidophilus NCFM, S. boulardii and E. coli EMO-associated animals brought the number of systemically injected bacteria down by approximately three logarithmic units, as did conventional animals. However, 90 minutes after injection of the pathogen, both S. boulardii and E.coli EMO-associated animals had not cleared the injected bacteria any further, while the MPS of L. acidophilus NCFM-associated mice continued to remove bacteria from the circulation. Similar results were seen when cyclophosphamide-treated conventional mice were associated with probiotics (Fig. 3). Hence, L. acidophilus NCFM-associated animals cleared bacteria with greatest efficiency (Fig. 3A). Mice associated with S. boulardii could only remove bacteria at a slightly higher rate than non-associated, cyclophosphamide-treated animals (Fig. 3B). Surprisingly, E. coli EMO-associated, cyclophosphamide-treated animals showed an even lower rate of clearance than the non-associated controls (Fig. 3C).
the systemic circulation more efficiently when the animals were mono-associated with *L. acidophilus* NCFM. Interestingly, conventional animals treated with cyclophosphamide (alkylating agent that blocks cell replication) and associated with *L. acidophilus* exhibited restored phagocytic capacity of the MPS. The mechanism of immunoregulation by the intestinal microflora is still unknown although it has been under study for a long time. Mìake et al. demonstrated the protective effect of *L. casei* and *L. acidophilus* against *Pseudomonas aeruginosa*. Tomioka et al. showed the same effect against *Listeria monocytogenes*. These and other researchers suggested that the protective effect of lactobacilli was afforded by activated macrophages, interferon production and accumulation of phagocytes that cleared the infecting bacteria.

The precise mechanisms of action of *L. acidophilus* NCFM in the MPS are still poorly understood, but the results of this work show us that it is more efficient than the other probiotics analyzed in this study. Maybe there is more than one mechanism involved in immunomodulation by probiotics since we have found different behavior for each probiotic studied herein.

The clearance test is a good assay to determine the phagocytic activity in vivo. Perdigon et al. used the clearance of carbon and demonstrated that mice treated with *L. casei* and *L. acidophilus* triggered the clearance of carbon particles from the blood more efficiently than mice who did not receive the microorganisms. Shahani et al. also used the carbon clearance test and showed that *L. acidophilus* and *S. thermophilus* accelerated the function of the reticuloendothelial system. In both cases they used conventional animals. We introduced germfree mice to demonstrate the direct effect of the probiotic on the host MPS. Moreover, we used bacterial clearance instead of carbon clearance. We believe the use of biological agents is more appropriate for studying augmentation of phagocytic activity in vivo since it involves complex antigens as in a bacteremia situation.

Our experiments showed that germfree animals are good tools for testing the influence of probiotics in MPS since we can study each one separately. We demonstrated the important role of intestinal microbiota and probiotics in the host MPS. However, the mechanisms of action of various probi-

**DISCUSSION AND CONCLUSION**

According to the results presented above, probiotics restore clearance activity in both germfree and immunosuppressed animals. The pathogenic *E. coli* B41 was cleared from

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Fig. 3: Clearance of *E. coli* B41 in cyclophosphamide-treated conventional mice associated with *L. acidophilus* (A), *S. boulardii* (B) or *E. coli* EMO (C).

Squares represent cyclophosphamide-treated, probiotic-associated animals, circles represent cyclophosphamide-treated, non-associated controls. Experiments were performed as described in the legend for Fig. 1. Each point represents the average of five mice. Vertical bars stand for standard deviations of the means. Data are from one representative experiment out of two performed. * indicate statistically significant difference (p<0.05).
are different and require further study. At the same time, the marked stimulative activity of *L. acidophilus* NCFM on the MPS under immunosuppressive conditions harbors important clinical potential.

ACKNOWLEDGMENTS

This work was supported by CNPq grant number 520100/95-6. We thank Ronilda Maria de Paula and Maria Helena Alves de Oliveira for the animal care. E.N. is supported by CAPES and L.Q.V. is supported by CNPq. G.I.P is supported by CNPq and FAPEMIG.

REFERENCES

SOME PARASITOLOGICAL AND IMMUNOLOGICAL ASPECTS OF THE EXPERIMENTAL INFECTION WITH TRYPANOSOMA CRUZI IN GERMFREE AND CONVENTIONAL RATS

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SUMMARY

The effects of experimental infection with Trypanosoma cruzi, Colombian strain, upon blood parasitaemia, tissue parasitism and trypanosome-specific IgG levels were examined in germfree (GF) and conventional (CV) 56 day old Wistar rats. The parasitaemia was more precocious and higher in the GF group. However, on histopathological examination, there was no difference between GF and CV animals. All animals survived. Anti-trypanosome IgG were present in statistically higher levels in GF than in CV rats (p<0.05), by day 87 after the infection. When GF and CV rats were reinfected, 73 days after the first infection, the IgG levels were lower by day 87 after the first infection, when compared with the levels obtained at day 56. This difference was statistically significant (p<0.05) only in the GF group. The relevance of these data are discussed.

INTRODUCTION

The studies of parasitic diseases under germfree (GF) conditions are important for understanding the host-parasite relationship, since interference of the associated microflora is excluded. As a consequence, the influence of intestinal microflora on the course of parasitic diseases can be evaluated. The complete life cycle of Trypanosoma cruzi, the aetiological agent of the American trypanosomiasis (Chagas' disease), was achieved under GF conditions (Resende et al., 1992). Experimental infection with T. cruzi has been reported to be more severe in terms of parasitaemia, mortality, and parasitism in GF than in conventional (CV) rats and mice (Silva et al., 1987). Other results showed that the reduced humoral immune response of GF mice just after infection may be responsible for the more severe parasitism when compared to CV mice (Fururah et al., 1991).

In this paper, the parasitism and some aspects of the humoral immune response in mature GF and CV rats experimentally infected with T. cruzi were evaluated.
Table 1: Epimastigote-binding IgG levels in conventional (CV) and germfree (GF) rats by day 87 after initial infection with Colombian strain of Trypanosoma cruzi.

<table>
<thead>
<tr>
<th>Group</th>
<th>First infection</th>
<th>Reinflection</th>
</tr>
</thead>
<tbody>
<tr>
<td>GF</td>
<td>0.700 ± 0.093a</td>
<td>0.398 ± 0.038b</td>
</tr>
<tr>
<td>CV</td>
<td>0.262 ± 0.088c</td>
<td>0.221 ± 0.073c</td>
</tr>
</tbody>
</table>

Reinfected animals received a second trypanosome challenge, 73 days after the initial infection. Rats received $12 \times 10^6$ blood form trypomastigotes, intraperitoneally, on days 0 and 73. Antibodies were determined by ELISA and results are reported as absorbance at 492 nm. Data are reported as means ± SEM for 3 rats in each group. Groups with the same letter were not statistically different (p<0.05).

METHODS

Colombian strain of *T. cruzi* was maintained in CV mice. The inoculum was obtained as described previously (Silva et al., 1987). Breeding nuclei of GF (LOF) Wistar rats were supplied by Dr. Morris Pollard, University of Notre Dame, Notre Dame IN, USA, and maintained in flexible plastic isolators. The CV animals were derived from the GF colony. Groups of 56 day old GF and CV rats, were inoculated intraperitoneally with $12 \times 10^6$ trypomastigotes. Blood trypomastigotes were counted daily as described by Brener (1962). In a first experiment, the CV and GF groups were anaesthetised on day 87 after the infection and blood was drawn from the axillary plexus. In a second experiment, GF and CV rats were reinfected 73 days after the first infection and the animals were killed as described above on day 87 after the first infection. Trypanosome-specific IgG in serum samples were identified by ELISA as described previously (Furarah et al., 1991). After death, tissue samples were fixed in 4% formaldehyde, processed for paraffin embedding and submitted to a histo-pathological examination. Data were evaluated statistically by analysis of variance and means were compared using the least significant difference test.

RESULTS AND DISCUSSION

The parasitaemia was more precocious and higher in the GF group. All the animals survived to the 71st day, when they were killed. On histopathological examination, no difference between the GF and CV animals could be detected. A discrete focal parasitism in the muscular and reticulendothelial system could be visualised in both groups. Previous data showed that the levels of parasitaemia were also higher when 21 day old GF rats were compared to CV animals of the same age. In this case, all GF and 80% of the CV rats died by day 19 after the infection. There was no parasitaemia in reinfected GF and CV animals, as described for CV mice.

The data in Table 1 show that circulating anti-trypanosome immunoglobulins of the IgG class in GF rats were present at significantly higher
levels than in CV animals at the term of the experiment (p<0.05). In both GF and CV reinfeeted animals, the IgG levels were lower when compared with the results obtained during the first infection. This difference was statistically significant only in the GF group (p<0.05). The course of infection with *T. cruzi* is marked by severe alterations of the immune response of the host in both humans and laboratory animal models. Thus, antibody production as well as specific and non-specific cellular immune responses are suppressed during the acute phase of the infection, but a trend towards immunological normalisation is seen during the chronic period (Jeng and Kierszenbaum, 1984). Because of the lack of contact with a microbial flora, GF animals have an underdeveloped but intact immune system. When activated by antigenic materials these immune mechanisms respond as well as, or even better than, their conventional counterparts (Pollard and Sharon, 1970). This fact could explain the higher anti-trypanosome IgG levels in GF rats when compared with CV animals, in a similar manner to that described during the infection of mice by *T. cruzi* (Furarah et al., 1991) and of rats by *T. lewisi* (Giannini, 1987).

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LITERATURE


INFLUENCE OF SOME BACTERIA FROM THE DUODENAL DOMINANT MICROBIOTA OF PATIENTS WITH SYMPTOMATIC GIARDIASIS IN GNOTOXENIC MICE EXPERIMENTALLY INFECTED WITH GIARDIA LAMBLIA


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Recent results show that intestinal microbiota is essential for the pathogenicity but not for the multiplication of Giardia lamblia in the intestinal lumen by day 10 after experimental infection in mice. The microbial components responsible for this phenomenon are not known. Six bacteria were isolated in an anaerobic chamber (85% N₂, 10% H₂, 5% CO₂) from the duodenal dominant microbiota of 3 patients with symptomatic giardiasis. Respiratory tests showed that all these microorganisms were facultative anaerobes. Each bacterium was monoassociated with a group (GN) of 21-day old germfree mice and reared in microisolators. Four days after the monoassociation, their fecal populations ranged from 1⁰ to 1⁹ CFU/g. At this time, all the groups were inoculated intragastrically with 2.5 x 1⁹ viable trophozoites of G. lamblia strain BT6. Two groups, germfree (GF) and G. muris-free (CV), respectively, were also infected and used as controls. Ten days later, all the animals were sacrificed and a histopathological examination performed. Higher pathological alterations were confirmed in CV mice when compared with GF animals. In contrast, there were no significant morphological differences between the GN groups which were histologically similar to the GF group.

INTRODUCTION

Giardia infection causes variable clinical manifestations ranging from asymptomatic or transient intestinal complaints that resolve spontaneously to severe long-standing disease with malabsorption and debilitation. 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 but recent results have shown that intestinal microbiota is essential for the genesis of the disorder. The presence of gut microbiota is also essential for the pathogenic expression of some other enteric protozoa such as Entamoeba histolytica, Eimeria ovinaidalis, E. tenella and E. falciformis. Working with E. histolytica, Mirelman and Bracha showed that lesions were observed only with axenic amoebae reassocaited with a Gram-negative bacterium. The microbial components and mechanisms responsible for the phenomenon involving G. lamblia are not known.

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

PATIENTS AND METHODS

Patients
Three children, aged 11 months, 2 and 7 years, treated for symptomatic giardiasis at the Federal University of Minas Gerais Hospital were the duodenal dominant micro-

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biota donors. Duodenal biopsies were exploited to obtain a sample of duodenal fluid. This material was diluted in a tube containing 1 ml of prereduced anaerobically sterilized dilution fluid (Ringer-PRAS) flushed with oxygen-free CO₂. The study was approved by the hospital scientific and ethics committee. The children participated in the study with the informed consent of their mothers.

Isolation of duodenal dominant microbiota

Within one hour of collection, the diluted duodenal fluid was introduced into an anaerobic chamber (Forma Scientific, Marietta, USA - 85% N₂, 10% H₂, 5% CO₂) and serially diluted in tubes containing prereduced buffered saline. Samples from 10⁻¹ to 10⁻⁶ dilutions were spread on Blood Agar supplemented with hemin and menadione and incubated at 37°C for 7 days. Then, viability counts were performed, using the adequate dilution, and morphologically different colonies were isolated. The isolates were submitted to a respiratory test.

Mice

Germfree 21-day old mice of both sexes were used as bacterial receptors in this work. The animals were housed in flexible plastic isolators (Class Biologically Clean Ltd., Madison, USA), handled according to established procedures⁸. The animals were fed ad libitum a commercial autoclavable diet (Nuvital, Curitiba, Brasil). Germfree mice associated with a filtered G. muris fecal microbiota, as described in a previous work presented in this Symposium, were used as the conventional controls. Experiments with gnotoxenic or filtered intestinal microbiota mice were carried out in microisolators (UNO Roestvaststaal B.V., Zevenaar, The Netherlands).

Association of germfree mice with duodenal isolates

The bacterial isolates were grown separately on supplemented Blood Agar for 48 hr at 37°C in an anaerobic chamber. Then, they were washed off with buffered saline and each bacterial isolate was associated immediately with a group of germfree mice. A 0.5 ml portion of these suspensions was used for inoculation of each animal by gastric intubation.

Giardia lamblia infection

Five days after the monoassociation, each group of gnotoxenic mice (GN) was inoculated intragastrically with 2.5 x 10⁵ viable trophozoites of G. lamblia strain BTU6/89/JMS. This strain was kindly supplied by Dr. M.I.T. Sogayar (Department of Parasitology, Paulista State University, Botucatu, Brasil). Two groups, germfree (GF) and G. muris-free (CV), respectively, were also infected and used as controls. Ten days later, all the animals were sacrificed by ether inhalation and subjected to histopathological examination.

Anatomopathological examination

After sacrifice, the small bowel was removed. A 1-cm segment was excised 10 cm from the gastroduodenal junction. The excised segment was opened longitudinally, oriented on filter paper, and fixed in 4% formaldehyde for histopathological examination.

Parasitological analysis

Quantitation of cysts in feces and trophozoites in the small bowel was performed as described by Roberts-Thomson et al⁹.

RESULTS AND DISCUSSION

Six bacteria were isolated in the anaerobic chamber from the duodenal dominant microbiota of patients with symptomatic giardiasis. One patient showed no bacteria in his duodenal fluid, even at the 10⁻⁴ dilution. The second, showed only one colonial morphology at a population level of 3.90 log CFU/ml. In the duodenal fluid of the third patient, five colonial morphologies were found ranging between 4.84 to 7.04 log CFU/ml. Respiratory tests showed that all these microorganisms were facultative anaerobes. Each bacterium was monoassociated with a group (GN) of 21-day old germfree mice and reared in microisolators. Five days after the monoassociation, their fecal populations ranged from 7.0 to 9.0 log CFU/g. Fig. 1 shows that higher pathological alterations were observed in CV mice (Fig. 1A) when compared with GF animals (Fig. 1B) confirming the results of Torres et al⁹. In contrast, there was no significant morphological differences between GN groups which were histologically similar to the GF group (Fig. 1C).
during the sequential colonization of the host, the protozoa is first stimulated for its pathogenicity in the lower portion of the small bowel (ileum) before reversion back to its normal pathological site (duodenum).

ACKNOWLEDGMENTS

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CONCLUSION

This work suggests that the bacteria responsible for the stimulation of G. lamblia pathogenicity are not present in the duodenal dominant microbiota. It is possible that
SOME PARASITOLOGICAL AND IMMUNOLOGICAL ASPECTS OF GERMFREE AND CONVENTIONAL MICE EXPERIMENTALLY INFECTED WITH GIARDIA LAMBLIA

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As is the case for other intestinal pathogenic protozoa, the gut microbiota is indispensable for the expression of Giardia lamblia pathogenicity appearing in mice by day 10 after experimental infection. Two groups of germfree (GF) and G. muris-free conventional (CV) mice, respectively, were inoculated intragastrically with 2.5 x 10^3 viable trophozoites of G. lamblia strain BT6 and sacrificed 10, 20 and 30 days after infection. The two groups became infected by day 3 and fecal cyst levels were similar in both CV and GF groups throughout the experiment (about 10^8 cysts/g feces). Fecal weight before and during the infection was higher in GF animals when compared with the CV group. Total and G. lamblia-specific IgA levels increased in the intestinal fluid during the infection and were higher in CV animals at all times. As expected, stronger pathological signs were observed in CV mice in comparison to the GF animals.

INTRODUCTION

Intestinal microbiota is known to have an enhancing effect on the virulence of various protozoa. The gut microbiota is also indispensable for the expression of Giardia lamblia pathogenicity in mice appearing by day 10 after experimental infection. Some hypotheses have been proposed on the mechanisms involved in this pathogenic stimulation by bacteria. Among them, some are related to changes due to axenization of: 1) the protozoa such as a possible decrease in adhesion (changes in superficial carbohydrates) or in invasive abilities (decrease of proteolytic activities) observed in protozoa freed of their bacterial endosymbiont; 2) the host such as the thicker intestinal mucin layer (physical and chemical barrier) observed in germfree animals. 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 possible other predisposing factors.

In this work, we followed some parasitological and immunological parameters during experimental infection of germfree and conventional mice with G. lamblia.

MATERIAL AND METHODS

Mice

Germfree (GF) 21 day-old mice of both sexes were used in this work. The animals were housed in flexible plastic isolators (Class Biologically Clean Ltd., Madison, USA), handled according to established procedures. The animals were fed ad libitum a commercial autoclavable diet (Nuvital, Curitiba, Brazil). Germfree mice associated with a filtered G. muris-free fecal microbiota, as described in a previous work presented in this Symposium, were used as the conventional (CV) controls. Experiments with GF and CV mice were carried out, respectively, in isolators and microisolators (UNO Roestvaststaal B.V., Zevenaar, The Netherlands).

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Giardia lamblia infection

GF and CV mice were inoculated intra-gastrically with 2.5 x 10^5 viable trophozoites of G. lamblia strain BTU6/89/JMS. This strain was kindly supplied by Dr. M.I.T. Sogayar (Department of Parasitology, UNESP, Botucatu, Brasil). Fecal weight was determined daily during a 2 hr period. Groups of 3 or 4 GF and CV animals were sacrificed by ether inhalation 0, 10, 20 and 30 days after the infection and subjected to histopathological examination.

Anatomopathological examination

After sacrifice, the small bowel was removed. A 1-cm segment was excised 10 cm from the gastroduodenal junction. The excised segment was opened longitudinally, orientated on filter paper, and fixed in 4% formaldehyde for histopathological examination.

Parasitological analysis

Quantitation of cysts in feces and trophozoites in the small bowel was performed daily as described by Roberts-Thomson et al. 10.

Immunoglobulin determination

Total and G. lamblia-specific immunoglobulins of class A were determined by ELISA in the intestinal fluid by our modification of the method described by Lim et al. 11. In this modification, dilution of intestinal contents was performed at a ratio of 0.5 g contents/2ml PBS. In the original method, intestinal contents were uniformly diluted in 2 ml PBS, pH 7.2, independently of their weight.

RESULTS AND DISCUSSION

The two groups became infected by day 3 and fecal cyst levels were similar in both CV and GF groups throughout the experiment (about 6.0 log cysts/g feces) (Fig. 1).

Fig. 1: Cyst number in feces from germfree (GF) or conventional (CV) mice experimentally infected with 2.5 x 10^5 viable trophozoites of Giardia lamblia strain BT-6.

Fig. 2: Fecal weight in feces from germfree (GF) or conventional (CV) mice experimentally infected with 2.5 x 10^5 viable trophozoites of Giardia lamblia strain BT-6.

Fig. 3: Giardia lamblia-specific IgA in intestinal fluid from germfree (GF) or conventional (CV) mice experimentally infected with 2.5 x 10^5 viable trophozoites of Giardia lamblia strain BT-6.
Fecal weight before and during the infection was higher in GF animals when compared with the CV group (Fig. 2). Water loss, and increasing fecal weight is well documented in axenic rodents. G. lamblia-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. Greater increase of total IgA in comparison to the G. lamblia-specific immunoglobulins could be due to secondary pathological insult from components of the intestinal microbiota. During the experiment, higher pathological alterations were confirmed in CV mice when compared with GF animals. But these differences decreased by day 30 of infection.

CONCLUSION

In spite of its underdeveloped immunological system, the GF animals did not develop the intestinal pathological alterations observed in their CV counterparts during experimental infection with G. lamblia. This fact reinforces the importance of bacterial components from intestinal microbiota as stimulating factors for protozoa pathogenicity. However, the histopathological differences between GF and CV animals seem to disappear in the course of infection.

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OBTAINING MICE ASSOCIATED WITH A FILTERED INTESTINAL MICROBIOTA FREE OF GIARDIA MURIS AND ITS USE AS AN ANIMAL MODEL FOR EXPERIMENTAL INFECTION WITH GIARDIA LAMBLIA


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In our laboratory, experimental infection of mice with Giardia lamblia has been difficult to achieve, since conventionally-reared animals are naturally infected with G. muris. Adult germfree NMRI mice of both sexes reared in isolators were used as receptors for a filtered intestinal microbiota free of G. muris. Feces from conventional NMRI mice naturally infected with G. muris were introduced into an anaerobic chamber and diluted in buffered saline. Then, this suspension was submitted to differential filtration through a 3 μm filter to obtain a fecal suspension containing intestinal bacterial microbiota (< 3 μm) free of G. muris (> 3 μm). This fecal suspension was inoculated intra-gastrically into germfree NMRI mice. Ten days later, examination of fecal samples from these animals showed the elimination of the protozoa. Two groups of these G. muris-free mice were inoculated intra-gastrically with 2.5 x 10⁸ viable trophozoites of G. lamblia strains BT6 and Portland, respectively. A third non-inoculated group was used as a control. Ten days later, histopathological examination of the small bowel of the two infected groups showed villus hypotrophy, high reactional hyperplasia of the lymphoid component and presence of parasitic trophozoites on the mucosa and in the intestinal lumen. No signs were observed in uninfected animals. These animal models were used in our laboratory to study the influence of components of the intestinal bacterial microbiota on the pathogenesis of G. lamblia.

INTRODUCTION

Recent results show that the intestinal microbiota is indispensable for the expression of the pathogenicity for many intestinal pathogenic protozoa¹,², but not for the multiplication of Giardia lamblia³. In our laboratory, experimental infection of mice with G. lamblia to study this phenomenon was difficult to achieve since, under our conditions, conventionally-reared animals are naturally infected with G. muris. Specific-pathogen free animals can be used, but their intestinal microbiological status must be proved adequate since, as stated above, the microbiota is involved in the pathogenesis of giardiasis. Relying on the size difference between bacteria and protozoa, we developed a method of obtaining mice with G. muris-free intestinal microbiota by differential filtration without loss of stimulation of G. lamblia pathogenicity.

MATERIAL AND METHODS

Mice

Germfree NMRI 21-day old mice (Central Institute for Animal Breeding, Hannover, Germany) of both sexes were used as filtered microbiota receptors in this work. The animals were housed in flexible plastic isolators (Class Biologically Clean Ltd., Madison, USA), handled according to established procedures⁴. The animals were fed ad libitum a commercial autoclavable diet (Nuvertical, Curitiba, Brasil). Conventional NMRI mice, derived from germfree animals, and kept in an open animal room for several
generations, were used as intestinal microbiota donors. Experiments with filtered intestinal microbiota mice were carried out in microisolators (UNO Roestvaststal B.V., Zevenaar, The Netherlands).

Production of G. muris-free mice

Feces from conventional NMRI mice naturally infected with G. muris were introduced into an anaerobic chamber (Forma Scientific, Marietta, USA - 85% N₂, 10% H₂, 5% CO₂) and diluted 1:25 in pre-reduced buffered saline. Then, this suspension was submitted to differential filtration through a 3 μm Millipore filter to obtain a fecal suspension containing intestinal bacterial microbiota (< 3 μm) free of G. muris (> 3 μm), and removed from the anaerobic chamber. A volume of 0.5 ml of this fecal suspension was immediately inoculated intragastrically into germfree NMRI mice maintained in microisolators.

Parasitological analysis

Examination of fecal samples for cysts and trophozoites was performed as described by Roberts-Thomson et al. before and after the experimental infection with G. lamblia.

Giardia lamblia infection and anatomopathological examination

Two groups of these G. muris-free (GMF) mice were inoculated intragastrically with 2.5 x 10⁵ viable trophozoites of G. lamblia strains BTU6/89/JMS (GMF1) and Portland (GMF2), respectively. These two strains were kindly supplied, respectively, by Dr. M.I.T. Sogayar (Department of Parasitology, Paulista State University, Botucatu, Brasil) and Dr. E.F. Silva (Department of Parasitology, Federal University of Minas Gerais, Belo Horizonte, Brasil). A third non-inoculated group was used as a control (GMF3). By day 10 after infection, all the animals were sacrificed by ether inhalation and the small bowel was removed. A 1-cm segment was excised 10 cm from the gastroduodenal junction. The excised segment was opened longitudinally, oriented on filter paper, and fixed in 4% formaldehyde for histopathological examination.

RESULTS AND DISCUSSION

Examination of fecal samples from germfree animals associated with the filtered microbiota showed the elimination of the protozoa. However, the loss though differential filtration of some components from the initial intestinal microbiota involved in the stimulating effect of G. lamblia pathogenicity was a possibility. Ten days after experimental oral infection with G. lamblia, histopathological examination of the small bowel of both groups infected with BT-6 (GMF1) or Portland (GMF2) strain of the

Fig. 1: Histopathological aspect of small intestinal mucosa from NMRI germfree mice associated with filtered intestinal microbiota. The animals were intragastrically challenged with 2.5 x 10⁵ viable trophozoites of BT-6 (a) or Portland (b) Giardia lamblia strains or not challenged (c). (R.A.S., 63 x).
protozoa showed villus hypertrophy, high reactional hyperplasia of the lymphoid component and presence of parasitic trophozoites on the mucosa and in the intestinal lumen (Figs. 1a and b). No signs were observed in uninfected animals (fig. 1c). These animal models were used in our laboratory to study the influence of components of the intestinal bacterial microbiota on the pathogenesis of *G. lamblia*.

**CONCLUSION**

The differential filtration used in this work allowed elimination of a natural protozoa from the murine intestinal microbiota without interfering with the bacterial components responsible for pathogenicity stimulation of *G. lamblia*.

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**REFERENCES**

ISOLATION AND PARTIAL IDENTIFICATION OF BACTERIA FROM THE RAT FECAL MICROBIOTA RESPONSIBLE FOR THE ANTAGONISM BY DIFFUSIBLE COMPOUNDS AGAINST SHIGELLA FLEXNERI AND SALMONELLA TYPHIMURIUM

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As in the case of Vibrio cholerae, the rodent intestinal microbiota exerts a potent antagonistic effect against Shigella flexneri and to a lesser extent against Salmonella typhimurium. We followed the appearance of an inhibitory halo against S. flexneri and S. typhimurium from the feces of 98 Wistar rats of both sexes at the ages of 15, 22 and 29 days. The percentage of rats showing the inhibitory halo against S. typhimurium or S. flexneri were the same independent of their age and sex (about 60 to 70%). Contrary to S. flexneri, there was no definite inhibitory halo for S. typhimurium but a delay of growth around the feces. Eight apparently distinct bacteria were isolated in an anaerobic chamber from the dominant fecal flora of a rat showing a halo against the bacteria. These microorganisms were associated intragastrically with germfree NIH mice for 5 days during which only 3 of the inoculated species reached dominant levels in the feces: a Lactobacillus sp, a Proteus mirabilis strain and a Bacteroides sp. In these gnotoxenic mice, there was a permissive barrier effect when the animals were challenged orally with 10⁹ viable S. flexneri cells. The results showed that, contrary to V. cholerae, the antagonism due to diffusible compounds and developed by the rat intestinal microbiota against S. flexneri and S. typhimurium was soon installed in young animals. This antagonism was a strong inhibition against S. flexneri and only a growth retardant for S. typhimurium.

INTRODUCTION

The role of the intestinal microbiota as a host defense was originally observed in rodents that had been treated with antibiotics and then challenged with Salmonella, Shigella⁵, or Vibrio cholerae⁶. In this way, the rodent intestinal microbiota exerts a potent antagonistic effect against Shigella flexneri and to a lesser extent against Salmonella typhimurium. Miller and Bohnhoff⁷ showed that disappearance of Bacteroides from the enteric microbiota seemed correlated with enhanced susceptibility to Salmonella infection. Utilizing individual components of the facultative anaerobic intestinal biota, Henges and Freter⁸ demonstrated that Escherichia coli and Aerobacter aerogenes were the most antagonistic against S. flexneri.

The possible mechanisms responsible for antagonism between bacteria in the intestinal ecosystem include competition for nutrients or adhesion sites and production of soluble substances (bacteriocins) or metabolites (volatile fatty acids). Lactic acid and volatile fatty acids are involved in the inhibition of S. typhimurium and S. flexneri in vitro⁹ but this effect has often failed to correlate with in vivo antagonism⁴. Results obtained by Ducluzeau et al.⁸ suggested that bacterial antagonism in the intestines can be related to the production, in vivo, by a Clostridium of an antibiotic substance active against S. flexneri. On the other hand, Ramaré et al.⁹ demonstrated that very potent antibacterial substances (in this case against Clostridium perfringens) can be produced through a mutual mechanism involv-
ing intestinal bacteria and host exocrine pancreatic secretions.

In this work, we studied some aspects of the antagonism against *S. typhimurium* and *S. flexneri* by diffusible compounds produced by the fecal microbiota from rats.

**MATERIAL AND METHODS**

**Animals**

Wistar rats of both sexes, 15 days old at the beginning of the experiment, were used to study the influence of age and sex. The animals were fed a commercial diet for rodents (Nuvital, Curitiba, Brasil). A rat showing an inhibitory halo against *S. flexneri* and *S. typhimurium* in its feces was used as the intestinal bacteria donor. Germfree NIH mice (Taconic, Germantown, USA) of both sexes were used as receptor animals for the screening of rat fecal bacteria responsible for the antagonistic compound production. The germfree animals were housed in flexible plastic isolators (Standard Safety Company, Pallatine, USA), handled according to established procedures. The animals were fed a commercial autoclavable diet (Nuvital, Curitiba, Brasil) *ad libitum*. Experiments with gnotobiotic mice were carried out in microisolators (UNO Roestvaststaal B.V., Zevenaar, The Netherlands).

**Experimental design**

To study the influence of age and sex, we followed the frequency of appearance of an inhibitory halo against *S. flexneri* and *S. typhimurium* from the feces of 98 Wistar rats (51 males and 47 females) at the age of 15, 22 and 29 days.

**Pathogenic bacteria**

The *Salmonella* and *Shigella* strains of human origin were obtained from Fundação Ezequiel Dias (FUNED, Belo Horizonte, Brasil) and Laboratório de Gastroenterologia Pediátrica (UFMG, Belo Horizonte, Brasil). The bacteria were identified as *Salmonella enteritidis* var. *typhimurium* and *Shigella flexneri*.

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**Fig. 1:** Inhibitory test against *Shigella flexneri* (A) or *Salmonella typhimurium* (B and C; 6 and 24hr of incubation time, respectively) in Brilliant Green Agar using feces from Wistar rats.
Detection of diffusible inhibitory substances by \textit{in vitro} test

Freshly collected feces from rats or mice were put in a Petri dish containing Brilliant Green Agar and incubated for 48hr at 4°C. Then, the plates were spread with a $10^{-2}$ dilution from a 24hr \textit{S. flexneri} or \textit{S. typhimurium} culture in Brain Heart Infusion and incubated for 24hr at 37°C when inhibitory halos were detected around the feces.

Screening of rat fecal bacteria producing inhibitory compounds against \textit{S. flexneri} and \textit{S. typhimurium}

The rat dominant fecal microbiota was obtained by decimal dilution in an anaerobic chamber (Forma Scientific, Marietta, USA - 85% N$_2$, 10% H$_2$, 5% CO$_2$) and plating onto Brain Heart Infusion (BHI) agar (supplemented with yeast extract, hemin and menadione) of the $10^2$ dilution. After incubation at 37°C for 7 days, the different colonial morphologies were counted, isolated and submitted to microscopic examination and respiratory and biochemical tests. Biochemical tests were carried out using BBL Crystal$^\text{TM}$ Identification System kits and the BBL Crystal ID System Electronic Codebook (Becton Dickinson Microbiology Systems, Cockeysville, USA).

Association of germfree mice with rat fecal bacteria

The bacterial isolates were grown separately on BHI agar for 48hr at 37°C in the anaerobic chamber. Then, they were washed off with buffered saline and associated with germfree mice immediately. A 0.5ml portion of these suspensions was used for inoculation of each animal by gastric intubation. \textit{In vitro} inhibitory test and \textit{S. flexneri} or \textit{S. typhimurium} challenges were made 5 days after the axenic mice were associated with rat fecal bacteria. A group a germfree mice was monoassociated only with \textit{Shigella flexneri} as a control.

![Fig. 2: Inhibitory test in Brilliant Green Agar using feces from germfree mice (A: \textit{Shigella flexneri}; B: \textit{Salmonella typhimurium}) or from mice octoassociated with facultative and strict anaerobic bacteria from the rat fecal microbiota (C: \textit{Shigella flexneri}).](image)
**S. flexneri** and **S. typhimurium** counts in feces from gnotobiatic mice

Freshly collected feces were submitted to decimal dilutions and plated on McConkey Agar. Incubation was carried out at 37°C and colonies were counted one day later.

**RESULTS AND DISCUSSION**

The percentage of rats showing inhibitory halo against **S. flexneri** was the same independent of their age and sex (about 60 to 70%). Contrary to **S. flexneri** (Fig. 1A), there was no definite inhibitory halo against **S. typhimurium** but a delay of growth around the feces (Figs. 1B and C). While generally all rats are resistant to intestinal colonization at least by **S. flexneri**, animals which did not show inhibitory halo around their feces probably had some other antagonistic mechanism against the bacteria developed by their microbiota. Eight apparently distinct bacteria were isolated in the anaerobic chamber from the dominant fecal flora of a rat showing halo against the bacteria. Respiratory tests showed that four of these bacteria were distinct Gram-negative strict anaerobic short rods (probably all of them Bacteroides) and four facultative anaerobes or microaerophilic (one Lactobacillus sp, one Enterococcus sp, one Proteus mirabilis and one unidentified Gram-positive rod). Fecal population levels were of about 9.0 and 8.0 log CFU/g for strict and facultative anaerobes, respectively (except for the Lactobacillus showing 9.49 log CFU/g). These microorganisms were associated intragastrically with germfree NIH mice for 5 days during which only 3 of the inoculated species reached dominant levels (about 10.0 log CFU/g) in the feces: the Lactobacillus sp, P. mirabilis and one of the Bacteroides. There was no halo against **S. flexneri** nor **S. typhimurium** from axenic animal feces (Figs. 2A and B). But a large inhibition zone against **S. flexneri** was observed around the feces from the octoxenic mice (Fig. 2C). However, there was only a permissive barrier effect when the animals were challenged orally with 10⁶ viable **S. flexneri** cells (Fig. 3). The number of pathogenic bacterial cells ranged around 8.0 and 10.0 log CFU/g of feces from, respectively, octoxenic and monoxenic control mice. But a higher survival was observed in octoxenic mice (100%) when compared to monoxenic animals (30%). Curiously, a **S. flexneri** growth stimulated zone was noted soon after the inhibitory halo from the octoxenic feces. This stimulating zone was also observed around the axenic feces. This phenomenon did not occur with **S. typhimurium**.

**CONCLUSION**

The results showed that, contrary to that against **V. cholerae**, the antagonism due to diffusible compounds developed by the rat intestinal microbiota against **S. flexneri** and **S. typhimurium** was soon installed in young
animals. This antagonism was a strong inhibition against *S. flexneri* and only a growth delay for *S. typhimurium.*

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ISOLATION AND PARTIAL IDENTIFICATION OF BACTERIA FROM THE RAT FECAL MICROBIOTA RESPONSIBLE FOR THE ANTAGONISM BY DIFFUSIBLE COMPOUNDS AGAINST VIBRIO CHOLERAE

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In a previous work, presented in this Symposium, some conditions for the production of antagonistic diffusible compounds from the rat intestinal microbiota against Vibrio cholerae were determined. Isolation of the bacteria producing these substances was performed, using 28 day old male Wistar rats fed a commercial rodent diet whose feces produced an inhibitory halo. The dominant fecal microbiota was obtained by decimal dilution in an anaerobic chamber, plating on Brain Heart Infusion agar of the 10^{-7} dilution and incubation for 7 days at 37°C. Three apparently different morphologies were isolated: a facultative anaerobe (FAN) and two strict anaerobes (SAN). These bacteria were associated with NIH germ-free mice reared in microisolators. Five days later, the inhibitory test showed an antagonistic halo around the feces of the trixenic mice but not for the axenic ones. Then, germfree mice groups were monoassociated with the FAN bacterium or diassociated with the 2 SAN for 5 days. Large inhibition zones were observed for the two groups but at different times (after 5 and 15 days of monoassociation for monoxenic and dixenic animals respectively). After oral challenge of both group with a 10^6 viable V. cholerae cell suspension, a permissive and a drastic barrier were observed, respectively, in FAN and SAN associated mice. Partial identification revealed Escherichia coli as the FAN strain. The two SAN bacteria were not identified but are distinct Gram positive cocci. These preliminary results suggest that the antagonistic effect of the rat intestinal microbiota against V. cholerae through diffusible substances was performed by more than one bacterial component at any given moment.

INTRODUCTION

The normal microbiota of the gastrointestinal tract is important in protection against gut infections by bacterial pathogens such as Salmonella typhimurium, Shigella flexneri and Vibrio cholerae. A study by Miller and Feeley1 dealt with the inhibitory activity of some intestinal bacteria against V. cholerae. Germfree mice were associated with mixtures of V. cholerae and aerobic and anaerobic bacteria commonly found in the intestinal tracts of mammals to determine which bacteria would eliminate V. cholerae from the mice. Not until a combination of Escherichia coli, Proteus mirabilis and Enterococcus faecalis was present in the intestinal tracts of the animals did V. cholerae disappear from the feces. However, the strain used for the mixture came from a different animal species and some of them did not belong to the dominant intestinal microbiota in eubiosis.

In a previous work, presented in this Symposium, some conditions for the production of antagonistic diffusible compounds from the rat intestinal microbiota against Vibrio cholerae were determined. Isolation and partial identification of the bacteria producing these substances were performed in the present work.

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MATERIAL AND METHODS

Animals

Twenty-eight day old male Wistar rats fed a commercial rodent diet whose fecal samples showed an inhibitory halo against *V. cholerae* in their feces were used as intestinal bacteria donors. Germfree NIH mice (Taconic, Germantown, USA) of both sexes were used as receptor animals for the screening of rat fecal bacteria responsible for the antagonistic compound production. The animals were housed in flexible plastic isolators (Standard Safety Company, Pallatine, USA), handled according to established procedures. The animals were fed *ad libitum* a commercial autoclavable diet (Nu- vital, Curitiba, Brasil). Experiments with gnotobiotic mice were carried out in microisolators (UNO Roestvaststaal B.V., Zevenar, The Netherlands).

Screening of rat fecal bacteria producing inhibitory compounds against *V. cholerae*

The rat dominant fecal microbiota was obtained by decimal dilution in an anaerobic chamber (Forma Scientific, Marietta, USA - 85% N₂, 10% H₂, 5% CO₂) and plating
onto Brain Heart Infusion (BHI) agar of the $10^{-7}$ dilution. After an incubation at 37°C for 7 days, the different colonial morphologies were isolated and submitted to microscopic examination and respiratory and biochemical tests. Biochemical tests were carried out using BBL Crystal™ Identification System kits and the BBL Crystal ID System Electronic Codebook (Becton Dickinson Microbiology Systems, Cockeysville, USA).

**Association of germfree mice with rat fecal bacteria**

The bacterial isolates were grown separately on BHI agar for 48hr at 37°C in the anaerobic chamber. Then, they were washed off with buffered saline and associated with germfree mice immediately. A 0.5ml aliquot of these suspensions was used for inoculation of each animal by gastric intubation. In *vitro* inhibitory tests were initiated 5 days after the germfree mice were associated with rat fecal bacteria. *V. cholerae* oral challenge ($10^8$ viable cells) was performed when an inhibitory halo was observed around the feces of the associated mice. Germfree mice challenged with the pathogenic bacteria were used as the control group.

**Detection of diffusible inhibitory substances by *in vitro* test**

Freshly collected feces from rats or mice were put in a Petri dish containing TCBS cholera medium and incubated for 48hr at 4°C. Then the plates were spread with a $10^{-1}$ dilution from a 24hr *V. cholerae* culture in BHI and incubated for 24hr at 37°C when inhibitory halos were detected around the feces.

**Vibrio cholerae counts**

After oral challenge, freshly collected feces from gnotoxenic mice were submitted daily to decimal dilutions and plated on TCBS cholera medium. Incubation was done at 37°C and colonies were counted one day later.

**RESULTS AND DISCUSSION**

From a previous work, presented in this Symposium, we determined that the frequency of appearance of the inhibitory halo against *V. cholerae* by diffusible compounds from rat feces was higher in male one-month old animals fed a rich protein diet when compared with females, younger males or rats fed a hypoproteic diet. Using these data, we selected a 28-day old male rat fed a commercial diet for rodents as fecal donor for the isolation of bacteria responsible for the antagonism against *V. cholerae*. From a $10^{-7}$ fecal dilution from this animal and after a 7-day incubation period at 37°C, we isolated 3 apparently different morphologies. Respiratory tests showed that one of these bacteria was a facultative anaerobe (FAN) and the others were strict anaerobes.
Fig. 3: Fecal population levels of *Vibrio cholerae* orally challenged in gnotoxenic mice previously associated with two strict anaerobic bacteria from the rat fecal microbiota or in germfree mice.

(SAN). These bacteria were associated with NIH germfree mice reared in microisolators. Five days later, the inhibitory test showed an antagonistic halo around the feces of the trixenic mice against the pathogenic bacterium (Fig. 1: 3AN). There was no halo from axenic animal feces (Fig. 1: AX). Then, two germfree mice groups were, respectively, monoassociated with the FAN bacterium or diassociated with the two SAN bacteria in separate microisolators. Large inhibition zones were observed for the two groups (Figs. 1: 1ANF and 2ANS) but at different times (after 5 and 15 days of association for monoxenic and dixenic animals, respectively). After oral challenge of monoxenic (Fig. 2) or dixenic (Fig. 3) mice with *V. cholerae*, the bacterial pathogen was, respectively, repressed or eliminated from the feces in about 2 weeks. Partial identification revealed *Escherichia coli* as being the FAN strain. The two SAN bacteria were not identified but are distinct Gram positive cocci. One of the SAN was an Extremely Oxygen Sensitive (EOS) strain. Whereas the rat is naturally resistant to intestinal colonization by *V. cholerae*, the animals which did not show inhibitory halo in this work probably used another mechanism to eliminate the bacterial pathogen.

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REFERENCES


CONCLUSION

These preliminary results suggest that the antagonistic effect of the rat intestinal microbiota against *V. cholerae* through diffusible substances was performed by more than one bacterial component at any given moment.
INFLUENCE OF AGE, SEX AND DIET ON THE ANTAGONISM AGAINST
VIBRIO CHOLERAE BY DIFFUSIBLE COMPOUNDS PRODUCED BY THE RAT
FECAL MICROBIOTA

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The rodent intestinal microbiota exerts a potent antagonistic effect against
Vibrio cholerae. Some microbial components responsible for this phenomenon
are known but not the mechanisms used or their functional conditions.
Production of diffusible substances is one of the possible mechanisms to
explain the antagonism. We followed the appearance of an inhibitory halo
against V. cholerae from the feces of 98 Wistar rats of both sexes at the ages of
10, 15 and 22 days. The animals were fed a commercial rodent diet. For the
inhibitory test, feces were included in TCBS cholera medium and incubated
for 48 hr at 4°C. Then, the plates were spread with a 10^3 dilution from a 24 hr
V. cholerae culture in Brain Heart Infusion and incubated for 24 hr at 37°C when
inhibitory halos were detected. The percentage of rats showing the halo from
their feces increased from 0% (10 days) to 35.3% (15 days) and finally 50.0% (22
days). Curiously, a lower frequency of appearance of this antagonism was
observed among females (0%, 23.53% and 35.29%) when compared with the
males (0%, 47.06% and 51.90%). In a separate experiment, when the rats (21
days old at the start) were fed a hypoproteic diet for 10 days, the inhibitory
halo disappeared completely. The potent antagonism developed by the rat
intestinal microbiota against V. cholerae seems to be due, in part, to diffusible
compounds and this phenomenon apparently depends on age, sex and nutrition
of the animals.

INTRODUCTION

The resistance of conventional rodents to intestinal colonization with human enteric
pathogens has been known for many years and the participation of the normal intesti-
nal microbiota in this function was well demonstrated by experiments using rodents
with an antibiotic-altered microbiota1 and gnotobiotic animals2. This antagonistic
phenomenon is potent but also fragile, being perturbed by factors such as drug ingestion,
administration and stress3. The possible mechanisms of this bacterial interaction include:
competition for nutrients or for adhesion sites; stimulation of peristalsis and local
immunity; production of inhibitory substances (bacteriocins) or metabolites (fatty
acids).

This work describes the influence of age, sex and nutritional hypoproteinaemia on the
production of a diffusible antagonistic substance against Vibrio cholerae in the feces of
rats.

MATERIAL AND METHODS

Animals and diets

Wistar rats of both sexes were used at 10 or 22 days old at the beginning of the exper-
iments on the influence of age and sex or hypoproteinaemia, respectively. To study the
influence of age and sex, the animals were fed a commercial rodent diet (Nuvital,
Curitiba, Brasil). For the nutritional experiment, the hypoproteic diet contained (g):
casein, 20.0; corn starch, 951.5; corn oil, 80.0;

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5963.
cellulose, 10.0; choline, 2.4; vitamin E, 0.1; mineral mixture, 40.0; vitamin mixture, 10.0. For the control diet, the same formulation was used except for casein (200g) and corn starch (827.5g). Control and hypoproteic diets were isocaloric.

Detection of diffusible inhibitory substances by in vitro test

Freshly collected feces from rats were put in a Petri dish containing TCBS cholera medium and incubated for 48hr at 4°C. Then, the plates were spread with a 10⁻¹ dilution from a 24hr V. cholerae culture in Brain Heart Infusion and incubated for 24hr at 37°C when inhibitory halos were detected around the feces.

Experimental design

To study the influence of age and sex, we followed the frequency of an inhibitory halo against V. cholerae from the feces of 98 Wistar rats (51 males and 47 females) at the age of 10, 15 and 22 days. For the nutritional experiment, hypoproteic and control groups were constituted of 11 and 6 animals, respectively.
The experimental diets were initiated at the age of 22 days and continued for one week (29 days old). After this time, both groups received the control diet for one more week (36 days). Inhibitory tests were performed at the age of 22, 29 and 36 days.

RESULTS AND DISCUSSION

Fig. 1 shows that the percentage of rats of both sexes showing inhibitory halo against *V. cholerae* from their feces increased from 0% (10 days) to 35.3% (15 days) and finally 50.0% (22 days). Curiously, a lower frequency of appearance of this antagonism was observed among females (0%, 23.53% and 35.29%) when compared with the males (0%, 47.06% and 61.90%) (Fig. 2). In a separate experiment, when the rats (22 days old; 63.64% exhibiting inhibitory halos to start with) were fed a hypoproteic diet for 7 days, the frequency of appearance of the inhibitory halo decreased to 14% (Fig. 3). After nutritional recovery with the control diet for one week, this frequency returned to 50.0%.

CONCLUSION

The potent antagonism developed by the rat intestinal microbiota against *V. cholerae* appears to be due, in part, to diffusible compounds and this phenomenon apparently depends on the age, sex and nutrition of the animals.

ACKNOWLEDGMENTS

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REFERENCES

THE GERMFREE ANIMAL AS A MODEL TO STUDY PARASITIC DISEASES

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Gnotobiotic (GN) mice have been used as models for the following diseases: schistosomiasis mansoni, American trypanosomiasis, leishmaniasis, and giardiasis. Germfree (GF) and conventional (CV) mice were infected with GF cercariae of Schistosoma mansoni. Biochemical indices related to granuloma formation and histopathological data suggest that the disease is less severe in GF than in CV mice even though the number of eggs recovered from the portal vein was similar for both groups of mice. Infection of GF and CV mice with Trypanosoma cruzi revealed that the disease is more severe in GF than in CV animals, according to the following parameters: parasitemia, mortality, and cell and tissue parasitism. Previous association of GN mice with Peptostreptococcus sp. led to a precocious mortality in GF mice on infection with T. cruzi. Cutaneous leishmaniasis (Leishmania amazonensis) was much more severe in CV than in GF mice. On the other hand, on inoculation with L. major in the foot, the lesion was six times bigger in GF than in CV mice after 13 weeks. On infection with Giardia lamblia, theecal cyst level was higher in GF than in CV group. The histopathological data revealed severe infection in CV mice whereas no alterations were found in the GF group. The afore-described results suggest that the use of GF animals in parasitic diseases may help to explain the host-parasite relationship.

INTRODUCTION

GF animals have been widely used as models in parasitic studies. One remarkable example of the importance of gnotobiology in parasitic diseases was given by amebiasis. It has been shown that pathogenicity of Entamoeba histolytica requires the presence of intestinal flora. In our laboratory, some work has been carried out on experimental schistosomiasis mansoni, American trypanosomiasis, leishmaniasis, and giardiasis in gnotobiotic animals.

SCHISTOSOMIASIS MANSONI

Germfree (GF) and conventional (CV) mice were infected with GF cercariae of Schistosoma mansoni. The cercariae were obtained from axenic snail (Biomphalaria glabrata) previously infected with miracidia aseptically isolated from livers of hamsters harboring S. mansoni. Biochemical indices related to granuloma formation and histopathological data suggest that the disease is less severe in GF than in CV mice even though the number of eggs recovered from the portal vein was similar for both groups of mice.

AMERICAN TRYpanosomiasis

(Chagas' Disease)

Infection of GF and CV mice with Trypanosoma cruzi revealed that the disease is more severe in GF than in CV animals as revealed by: (1) earlier and more intense parasitemia, (2) more precocious mortality, (3) twice enlarged spleen, (4) more intense cell and tissue parasitism, (5) visceral signs of cardiac failure. GF mice were associated with strict anaerobic bacteria (Peptostreptococcus sp., Bacteroides fragilis or Clostridium sp.) 10 days before the intraperitoneal infection with T. cruzi. The effects of the associations on parasitemia and mortality are
shown in Figs. 1 and 2, respectively. All colonization with associations of microorganisms led to precocious mortality upon infection with *T. cruzi*. *Peptostreptococcus* sp. produced the most remarkable result: a very low parasitemia and an early mortality. Since these microorganisms are normal residents of human intestinal flora, it may be inferred that this flora may play an important role in the susceptibility and severity of Chagas' disease. Determination of the immunoglobulin levels revealed that there was a three-fold increase in epimastigote-specific IgM and IgG in GF but not in CV mice (Figs. 3 and 4). These figures also show that the CV mice has a high level of *Trypanosoma*-specific IgM and IgG probably due to cross-reaction with intestinal microbiota.

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**Fig. 1**: Effect of association of strict anaerobic bacteria on parasitemia in germfree mice. Mice were inoculated intragastrically with bacteria, or not, and 10 days later were infected with 5000 trypanastigotes of the CL strain of *Trypanosoma cruzi*. *Bacteroides fragilis* (N=4), *Peptostreptococcus* sp. (N=10), *Clostridium* sp. (N=4), germfree (N=6).

**Fig. 2**: Survival of germfree mice associated with anaerobic bacteria after inoculation with *Trypanosoma cruzi*. *Bacteroides fragilis* (N=4), *Peptostreptococcus* sp. (N=10), *Clostridium* sp. (N=4), germfree (N=6).

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LEISHMANIASIS

Cutaneous leishmaniasis was much more severe in CV than in GF or GN (monocontaminated with Aspergillus sp.) mice as revealed by macro and microscopic examination. Leishmania amazonensis was inoculated subcutaneously at the root of the tail. Extensive ulceration occurred in all 16 infected CV mice whereas only one out of 15 GF animals showed a small ulcer. On the other hand, on inoculation with L. major in the foot, the lesion was six times bigger in GF than in CV mice after 13 weeks.
GIARDIASIS

On infection with Giardia lamblia, the fecal cyst level was higher in the GF than in the CV group (Fig. 5). The histopathological data revealed an intense infiltration in the lamina propria and high reactional hyperplasia of the lymphoid component were observed in the CV group. No alterations were found in the GF group. These results suggest that the intestinal microflora is indispensable for the expression of the pathogenicity but not for the multiplication of G. lamblia in the intestinal lumen.

CONCLUSION

The GF animal represents an excellent tool to study the host-parasite relationship. Some parasites elicit more severe disease in GF than in CV animals. Other parasites, even belonging to the same genre, demonstrated opposite behavior.

ACKNOWLEDGMENT

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8. Referência de Leda Leishmania.

INFLUENCE OF HYPO- AND HYPERPROTEIC DIETS ON SOME PARASITOLOGICAL AND IMMUNOLOGICAL ASPECTS OF EXPERIMENTAL CHAGAS' DISEASE IN GERMFREE AND CONVENTIONAL MICE


SUMMARY

The effects of 5 or 30% casein diets (hypo or hyperproteic, respectively) on some parasitological data and trypanosome-specific immunoglobulins of the IgG and IgM classes were studied in germfree (GF) and conventional (CV) CFW (LOB) mice. In the CV and GF groups, the highest parasitaemia was found in mice fed, respectively, on hyper- and hypoproteic diets. Histopathological examinations showed more intense cellular and tissue parasitism in GF than in CV mice. There was an increase in anti-trypanosome IgG and IgM levels in all GF and CV groups during the acute phase of the disease, except for the hypoproteic GF group. IgG and IgM levels were higher in the hyperproteic groups for both GF and CV animals. The significance of these data are discussed.

INTRODUCTION

Chagas' disease affects a wide variety of human population throughout the geographical areas of malnutrition in South America and it is reasonable to assume that a large number of individuals suffer from both malnutrition and Chagas' disease.

The nutritional status of the host may affect its relationship with the parasite (Keusch and Farthing, 1986). In conventional (CV) animals, a nutritional insult may either aggravate or ameliorate a disease. The reaction depends on the kind of deficiency and on the pathogen. In a series of papers, the effect of deficiencies of thiamine, panthotenate, pyridoxine, vitamin A, protein and zinc on the evolution of experimental American trypanosomiasis in rats was studied (Scrimshaw et al., 1968).

Normal intestinal microflora could be another factor influencing the course of a parasitic disease. Experimental Chagas' disease is much more severe in germfree (GF) than in CV mice and rats (Silva et al., 1987).

In this paper, the influence of protein level in the diet and intestinal microflora on the infection by Trypanosoma cruzi was studied in GF and CV mice fed either hypo- or hyperproteic diets.
Table 1: Epimastigote-binding IgG levels in conventional (CV) and germfree (GF) mice fed hypo- or hyperproteic diets by days 7 and 14 after infection with CL strain of *Trypanosoma cruzi*.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Days after infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7</td>
</tr>
<tr>
<td>GF hypoproteic</td>
<td>0.793±0.128a</td>
</tr>
<tr>
<td>GF hyperproteic</td>
<td>1.311±0.688*</td>
</tr>
<tr>
<td>CV hypoproteic</td>
<td>0.808±0.204</td>
</tr>
<tr>
<td>CV hyperproteic</td>
<td>1.079±0.183</td>
</tr>
</tbody>
</table>

Mice received 5x10³ blood forms of trypomastigotes, after 28 days on diets.
Antibodies were determined by ELISA and results are reported as absorption at 492 nm.
Data are reported as means ± SDEV for five mice in each group.
*a*: statistical difference (p<0.05) between nutritional groups with the same microbial status (GF or CV) and day of infection.

*": statistical difference (p<0.05) between GF and CV groups with the same nutritional status (hypo- or hyperproteic) and day of infection.

**METHODS**

CL strain of *T. cruzi* was maintained in CV mice. The inoculum was obtained as described previously (Silva et al., 1987). Breeding nuclei of GF (LOB) CFW mice were supplied by Dr. Morris Pollard, University of Notre Dame, Notre Dame IN, USA, and maintained in flexible plastic isolators. The CV animals were derived from the GF colony. Animals were 21 days old when experiments started: GF and CV mice received the diet during 28 days, and then were inoculated intraperitoneally with 5x10³ trypomastigotes. Blood trypomastigotes were counted daily as described by Brener (1962). On days 7 and 14 after the infection, five animals of each CV and GF groups fed the different diets were anaesthetised and blood was drawn from the axillary plexus. Trypanosome-specific IgG and IgM in serum samples were identified by ELISA as described previously (Furarah et al., 1991). After death, tissue samples were fixed in 4% formaldehyde, processed for paraffin embedding and submitted to histopathologic examination. Data were evaluated statistically by analysis of variance and means were compared using the least significant difference test.

**RESULTS AND DISCUSSION**

Previous results showed that protein deficiency affected less the GF than the CV mice as revealed by weight evolution and blood parameters (Cintra et al., 1990). The parasitaemia was higher in the GF hypoproteic group and lower in CV hypoproteic group (data not shown). The parasitaemia was nearly the same in CV and GF hyperproteic groups.
Table 2: Epimastigote-binding IgM levels in conventional (CV) and germfree (GF) mice fed hypo- or hyperproteic diets by days 7 and 14 after infection with CL strain of *Trypanosoma cruzi*.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Days after infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7</td>
</tr>
<tr>
<td>GF hypoproteic</td>
<td>0.439±0.207*</td>
</tr>
<tr>
<td>GF hyperproteic</td>
<td>0.596±0.077</td>
</tr>
<tr>
<td>CV hypoproteic</td>
<td>0.157±0.043a</td>
</tr>
<tr>
<td>CV hyperproteic</td>
<td>0.456±0.098</td>
</tr>
</tbody>
</table>

Mice received 5x10³ blood forms of trypomastigotes after 28 days on diets. Antibodies were determined by ELISA and results are reported as absorption at 492 nm. Data are reported as means ± SDEV for five mice in each group.

a*: statistical difference (p<0.05) between nutritional groups with the same microbial status (GF or CV) and day of infection.

*: statistical difference (p<0.05) between GF and CV groups with the same nutritional status (hypo- or hyperproteic) and day of infection.

with levels between the hypoproteic groups. On the histopathological examination, a more intense cellular and tissue parasitism was observed in GF than in CV mice. The nutritional status did not affect the histopathological picture. Data obtained by Yaeger and Miller (*Scrimshaw et al.*, 1968) showed higher parasitaemia, tissue destruction and mortality in rats fed a hypoproteic diet.

Table 1 shows that there was an increase in anti-trypanosome IgG levels in all GF and CV groups during the acute phase of the disease, except for the hypoproteic GF group. An increase in IgM levels was observed in all groups (Table 2). IgG and IgM levels were higher in the hyperproteic groups for both GF and CV animals. In relation to the microbial status, GF animals had a higher response during the acute phase for trypanosome-specific IgM and lower for IgG, independently of the nutritional status. Generally, the effects of protein malnutrition on humoral immunity are expected to be less severe that on cell-mediated immunity. B cell numbers and immunoglobulin levels are not affected significantly, but the antibody response may vary with the antigen and the form it is presented (*Terr et al.*, 1991).

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LITERATURE


USE OF AN *ESCHERICHIA COLI* AS PROBIOTIC FOR PIGLETS

P.C.R. ARAUJO, R.D. NARDI, C. MARTINS, P. RAIBAUD, Y. DUVAL-IFLAH, and J.R. NICOLI

SUMMARY

Probiotics are live microorganisms inoculated or fed to human and animals to obtain protection against enteric infections or increased zootecchnical performances. In this work, nine piglets were inoculated orally at birth (weight: 1.8±0.1 kg) with 10⁶ viable cells of a plasmid-free *Escherichia coli* strain. The control group consisted of ten piglets (weight at birth: 1.8±0.2 kg) which were inoculated with saline. Weight gain was determined during all the experiment. Weights of the *E. coli*-inoculated piglets were always higher than those of the non-inoculated group from weaning to the end of the experiment. At slaughter, the difference between the experimental group (89.6±2.8 kg) and the control group (78.3±3.9 kg) was statistically significant (p<0.01). The main difference between the *E. coli* studied in this work and the classical probiotics used in veterinary medicine was its utilisation in a single dose at the birth.

INTRODUCTION

After natural birth, new-born piglets have a large and mixed microbial population established in their intestinal tract. These microbes come from the mother and from the environment without selection. The sow is a major source of enteric pathogens including enteropathogenic *Escherichia coli* (Ducluzeau, 1985). Antagonisms among isogenic *E. coli* strains in the digestive tract have been evidenced (Onderdonk, 1981). Other results indicate that *in vivo* intraspecific interactions may also occur between strains of *E. coli* which have no parental relationships and which are of different animal origin. As an example, plasmid-free human *E. coli*, strain EMO, was shown to exert a barrier effect against a porcine plasmid-bearing enterotoxigenic *E. coli* strain in gnotobiotic mice (Duval-Iflah, 1983).

In this work, the possible protection of piglets against diarrhoea by a prior inoculation with the non-pathogenic *E. coli* EMO was investigated.
Figure 1: Weight gain in piglets inoculated intragastrically (I) or not (NI) with $10^6$ *Escherichia coli* EMO viable cells within 24 hrs after birth. D = weaning; C = nursery; R = growth; A = slaughter.
*: statistically different (p<0.01).

**METHODS**

**Bacteria**

A plasmid-free *Escherichia coli* (EMO) isolated from human stools was used (*Duval-Iflah*, 1983). One millilitre of an 18 hr brain heart infusion culture, diluted to 10%/ml, was administered intragastrically to piglets within 24 hrs of life (experimental group). A heat-killed preparation was inoculated in the control group.

**Groups**

Nineteen piglets of both sex, weighing about 1.8 kg at birth, were divided in two groups of nine (experimental) and ten (control) animals, respectively. They were fed on SOCIL farm rations appropriate for each growth period from weaning to slaughter.

**Analysis**

Animal weight was determined at birth, weaning, after nursery stay, and at slaughter.

**Statistical analysis**

The data were evaluated statistically by the Student's test using an EPISTAT program.

**RESULTS AND DISCUSSION**

One of the major problems in the production of meat from pigs is the pre-weaning mortality of about 20%. The major cause of death is diarrhoea (41%) and the most important causative agent is enterotoxigenic *E. coli* involved from 22% (Canada) to 82% (Australia) of the cases (*Jonsson and Conway*, 1992).

The feeding of preparations containing certain microorganisms to mammals and birds of commercial interest has received increasing attention during recent years. These living microbial preparations, called probiotics, were used to improve the per-
formance and disease resistance of these animals. Generally, the efficiency of these probiotics depends on the continuous presence in the diet. For this reason, probiotics must be resistant to the preparation of pelleted ration. However, previous work showed that an oral inoculation soon after birth of a K-88 strain of E. coli that does not produce enterotoxin afforded a considerable degree of protection in baby pigs against a K-88 enterotoxin producing strain of E. coli (Davidson and Hirsch, 1976). The authors concluded that this protection was due to the competition for combining sites on the cells of the small intestine.

In our experiment, E. coli EMO was administered intragastrically in a single dose soon after birth. Figure 1 shows that after weaning, the weight gain of experimental animals was higher than that of the control group and this difference (14.4%) was statistically significant at slaughter (p<0.01). There was no mortality in the two groups. A lower diarrhoea frequency in the experimental group could perhaps be responsible for the better zootechnical performance of these animals. A higher food efficiency due to enzymatic mechanisms could be another explanation for the observed difference. Studies which involve challenge with enteropathogenic E. coli strains in piglets inoculated with E. coli EMO are in course in our laboratory.

The main results obtained in this work are: 1) an E. coli isolated from human faeces was beneficial when inoculated intragastrically in piglets; 2) this beneficial effect was obtained with a single inoculum, soon after birth.

ACKNOWLEDGEMENTS

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LITERATURE

ORAL INOCULATION OF ESCHERICHIA COLI EMO IN HUMAN NEW-BORN S AS A PROBIOTIC AGAINST DIARRHOEA DURING THE FIRST YEAR OF LIFE


SUMMARY

Studies in mice, piglets and human neonates showed that a plasmid-free Escherichia coli (EMO strain) inhibited the further establishment of plasmid-carrying E. coli. The E. coli EMO strain was isolated from the faecal flora of a healthy adult, was non-toxigenic in vivo and in vitro and sensitive to all usual antibiotics. This work was undertaken to determine whether a precocious implantation of EMO into human new-borns would reduce the frequency of diarrhoea during a one year period. Fifty one healthy new-borns (experimental group) were inoculated oro-gastrically, within 2 hr after birth, with $10^6$ E. coli cells. In a control group, fifty one new-borns were inoculated with the heat-killed bacteria. New-borns receiving viable or killed E. coli EMO were chosen at random. The experiment was double-blind. During the one year period, the experimental group showed a 48% (23/48) diarrhoea frequency whereas in the control group this frequency was 73% (35/48). These values were statistically different ($p=0.037$), showing a protective effect against diarrhoea obtained through the inoculation of E. coli EMO in human new-borns soon after birth.

INTRODUCTION

In Brazil, infant mortality is a serious health problem, particularly in the low socio-economic population. This mortality is mainly due to diarrhoea and enteropathogenic Escherichia coli is responsible for 25 to 30% of the total cases. If components of the normal intestinal microflora contribute to host resistance to gastrointestinal infection, a practical application of this idea would be possible. The successful implantation of bacterial strains which are antagonistic to pathogens or potential pathogens in adult humans is likely to be extremely difficult. The adult gastrointestinal tract already harbours a normal microflora which will prevent the establishment of either a pathogenic or a non-pathogenic strain. Inoculation of the neonate's alimentary canal with members of the normal microflora
Table 1: Mean time of exclusive or partial breast-feeding during the first year of life in new-born groups inoculated with live (Experimental) or heat-killed (Control) *Escherichia coli* EMO.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean Time (months)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exclusive</td>
<td>Partial</td>
</tr>
<tr>
<td>Experimental</td>
<td>1.6</td>
<td>7.2</td>
</tr>
<tr>
<td>Control</td>
<td>1.7</td>
<td>7.5</td>
</tr>
</tbody>
</table>

may be more successful. The digestive tract of the human infant is sterile at birth and is colonised by some bacterial genera within less than 48 hr. Regardless of animal species, *E. coli* or enteric streptococci or both are almost universally the first organisms to appear in the colon (Moreau et al., 1986). Duval-Iflah and colleagues (1982) have achieved some success in reducing the number of antibiotic-resistant *E. coli* in infant faeces by inoculating the children, just after birth, with a plasmid-free human *E. coli* (EMO) which suppresses the multiplication of plasmid-bearing strains in the intestinal tract.

This work was undertaken to determine whether a precocious implantation of EMO into human new-borns might reduce the acute diarrhoea frequency during a one year period.

**METHODS**

**Bacteria**

A plasmid-free *Escherichia coli* (EMO) isolated from human stools was used (Duval-Iflah, 1982). One ml of an 18 hr brain heart infusion culture, diluted to 10⁶ viable cells/ml was administered to the experimental group. Heat-killed bacteria was inoculated in the control group. The administration was made orally to new-borns, within 2 hr of life.

**Patients**

One hundred and two new-borns were randomised into two groups of 51 each. Distribution of sex was similar in the two groups (24 and 25 males and 27 and 26 females, respectively, for experimental and control groups). All the patients were born in the Hospital das Clínicas, Faculdade de Medicina da Universidade Federal de Minas Gerais (UFMG), Belo Horizonte MG, Brazil. These patients were selected based on four criteria: pregnancy and birth without intercurrent diseases, parent’s authorisation and weight above 2.5 kg. A socio-economic level score (SELS) was performed for each new-born family, based on criteria such as: basic sanitation, availability of treated water at home, number of rooms at home, parent’s profession, etc. The SELS were similar in both groups. The experiment was approved by the Ethical Council of the Medical School.

**Clinical analysis**

During the one year period, clinical controls of all the infants were made monthly. Additional controls were made in case of intercurrent disease. Exclusive or partial breast-feeding were registered. Whenever diarrhoea occurred it was characterised.
Table 2: Occurrence of acute diarrhoea during the first year of life in new-born groups inoculated with live (Experimental) or heat-killed (Control) *Escherichia coli* EMO.

<table>
<thead>
<tr>
<th>Diarrhoea*</th>
<th>Experimental</th>
<th>Control</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>23**</td>
<td>34</td>
<td>57</td>
</tr>
<tr>
<td>Negative</td>
<td>25</td>
<td>14</td>
<td>39</td>
</tr>
<tr>
<td>Total</td>
<td>48</td>
<td>48</td>
<td>96</td>
</tr>
</tbody>
</table>

* : New-born which presented at least one acute diarrhoea episode during the first year of life.
** : Statistically different from the control group (p=0.037).

Acute diarrhoea was identified as a drastic change in the frequency of evacuation of liquid faeces, with three or more episodes daily. Protection ratio (*DuPont et al.*, 1987) was defined as:

\[
\text{Protection ratio (\%) = \frac{FDC \ - \ FDE}{FDC}}
\]

where FDC = % of diarrhoea in the control group, and FDE = % of diarrhoea in the experimental group.

Statistical analysis

Data were loaded on the EPI.INFO software (*Dean et al.*, 1990). For the analysis of frequency distribution, \(\chi^2\) test with Yates correction was used; Fischer exact test was applied whenever necessary. Variance analysis was used for comparison between means. Life Table method was used for evaluative analysis of the results through Log.Rank test for the comparison between the two curves (*Gustafsson*, 1987).

RESULTS AND DISCUSSION

During the experiment, six patients were excluded from the analysis. One new-born deceased (septicemia) in the control group and five patients (2 and 3 from control and experimental groups, respectively) could not be observed in spite of several attempts to locate them. As can be seen in Table 1, the mean times of exclusive and partial breast-feeding were similar in both groups. Table 2 shows that at the end of the experiment, the neonates of the experimental group presented a statistically lower (p=0.037) diarrhoea frequency (48%) than the control group (73%). The protection ratio of 32.3% was similar to the results obtained with *Lactobacillus GG* (*Oksanen*, 1990). Figure 1 shows that using another statistical treatment (adaptation of the Log.Rank survival curves test), 53% and 33% (p=0.040) of the infants in the experimental and control groups, respectively, did not have acute diarrhoea episodes after one year. Curiously, the difference between the two groups appeared only after six months of life. The final difference between the two groups was 20%. It should be pointed out that this percentage corresponds, approximately, to the diarrhoea frequency caused by enteropathogenic *E. coli*. 303
Figure 1: Diarrhoea curves during the first year of life in new-born groups inoculated with live (Experimental) or heat-killed (Control) *Escherichia coli* EMO. Log.Rank test showed that diarrhoea curve in the experimental group was statistically different from that of the control group (p=0.040).

This work showed that a relative protective effect against acute diarrhoea could be obtained through the inoculation of *E. coli* EMO in human new-borns soon after birth.

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LITERATURE


