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Protein malnutrition and metronidazole induced intestinal bacterial translocation in rats

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This study was designed to assess the effects of protein malnutrition (PM) associated with antibiotic on growth weight, cecal bacterial overgrowth and enterobacteria translocation. Eighteen Gnotobiotic young Wistar rats (135 ± 2.35 g) were treated orally with antibiotic and submitted to dietary restriction based on maize diet in order to determine gram-negative enteric overgrowth in the ceca, thereby promoting the translocation of these bacteria from the gastrointestinal tract. The control group (n= 6) was fed conventional diet, the malnourished rats (n= 6) were fed only maize (10 g/rat/day) and the metronidazole treated malnourished rats (n= 6) were fed only maize (10 g/rat/day) and treated orally with metronidazole (1 mg/ml) for 10 days. The PM associated or not with metronidazole increased the enteric bacilli populations in the ceca and promotes their translocation to the mesenteric lymph nodes. There was a direct relationship between cecal bacterial overgrowth, the numbers of viable enterobacteria of this strain present in the mesenteric lymph nodes (MLN) and protein malnutrition associated with metronidazole. This study provides confirmation that PM and PM associated with metronidazole decrease body weight and promotes cecal bacterial overgrowth and enterobacteria translocation in the MLN and liver.

Key words: Rats, enteric bacilli translocation, protein malnutrition, antibiotic, mesenteric lymph nodes.

INTRODUCTION

Protein malnutrition (PM) is the principal nutritional problem in most industrial areas of the world. Proliferation of bacteria in the small intestine to higher concentrations than normally present has been consistently reported in patients with this disease. The gastrointestinal tract serves as a potent barrier that prevents luminal bacteria from entering the host. This barrier function is maintained by a well-balanced intestinal flora, an unaltered permeability of the intestinal mucosa, and a normal functioning immune system. Furthermore, the intestinal mucosa, in addition to its role in nutrient absorption, function as a

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major barrier that prevents bacterial colonizing the gut from invading systemic organs and tissues.

An imbalance of intestinal microflora and overgrowth by gram-negative facultative bacteria through administration of antibiotic enhances translocation (Berg, 1992; Mikelsaar and Türi, 1990). In animal models, malnutrition is associated with villous atrophy and loss of intestinal weight in variable severity (Welsh et al., 1998). These disturbances can facilitate the passage of viable bacteria through the intestinal barrier via the lamina propria and other organs as the liver. This phenomenon is definite intestinal bacterial translocation from the gut (Berg, 1999). It is generally agreed that gram-negative faculta-

tive bacteria translocate more easily than anaerobes and gram-positive bacteria (Wells, 1990).

Table 1. Composition of the maize diet (% per 100 g).

Humidity	Protein	Grease	Ashes	Fiber	Carbonhydrate	Calories
12.2	8.4	4.5	1.1	1.3	73.9	370

Table 2. Standard conventional diet for rats.

	Cereals and product starch	
Ingredients	Co product of the transformation of cereals	
	Oil cakes and other nitrogenized products of vegetable origin	
	Nitrogenized product of origin	
	Mineral substances	
	Oils and grease	
Components Analytical	Gross products	23%
	Rough fat content	0.43%
	Crude fiber	4%
	Moisture	2%
	Rough ashes	5.5%
	Insoluble ashes in HCl	2%
	Copper	30 mg/kg
Vitamins	A	12,000UI/kg
	D3	3,000 UI/kg
	E	30 mg/kg

Caloric value = 2900 kcal /kg. Ration day laborer = 18 – 25 g.

rupted experimentally or due to disease, viable bacteria may transverse the gut mucosa and spread to the mesenteric lymph nodes (MLN) or more distant organs such as the liver and spleen, a process termed bacterial translocation (Deitch et al., 1987). Factors that promote bacterial translocation from the gut include disruption of the indigenous gastrointestinal (GI) microflora leading to bacterial overgrowth, impaired host immune defenses, and physical disruption of the gut mucosal barrier (Deitch., 1985).

Nowadays, metronidazole (2-methyl-5-nitroimidazole-1-ethanol) is widely used to treat protozoan diseases and against mainly anaerobic bacteria, including *Bacteroides* (Freeman et al., 1997). Animal and human studies have also demonstrated reversal and prevention of steatohepatitis following metronidazole treatment for small intestinal bacterial overgrowth (Reza Khoshini et al., 2008). Berg (1981) shows that oral treatment of mice with penicillin, clindamycin or metronidazole for only 4 days disrupts the normal flora ecology, allowing an overgrowth in the ceca of the gram-negative enteric bacilli and promoting their translocation to the mesenteric lymph

If one or more of these protective mechanisms are disnodes. They demonstrated that abnormally high population levels of certain bacteria in the GI tract promote translocation of these bacteria from the GI tract to the MLN and possibly other organs. A long-term therapy and high metronidazole concentration could interfere with microbial pathogenicity, resulting in changes to host bacterium relationship and involves a selection of the resistant stocks (Bourillon et al., 1978; Galuppo et al., 2000).

The objective of this work was thus to assess the effects of protein malnutrition alone or associated with metronidazole on body weight loss, intestinal microbial ecology and translocation of enterobacteria from the small intestinal tract to MLN and liver in rats.

MATERIALS AND METHODS

Animals and diets

Eighteen Gnotobiotic young Wistar rats weighing 135 ± 2.35 g (Charles River France – St Aubin les Elbeuf, France) 6 - 8 weeks old, at the beginning of the experiment were housed individually in stainless steel wire cages at 22 ± 2°C, on a 12 h light-dark cycle.

After 3 days of acclimatization, 18 rats were divided into three groups of six rats; they had free access to water *ad libitum* and standard pellet food (UAR, Villemoisson-sur- Orge, France), a conventionally diet containing proteins, fat, carbohydrate, vitamin and minerals (Tables 1 and 2).
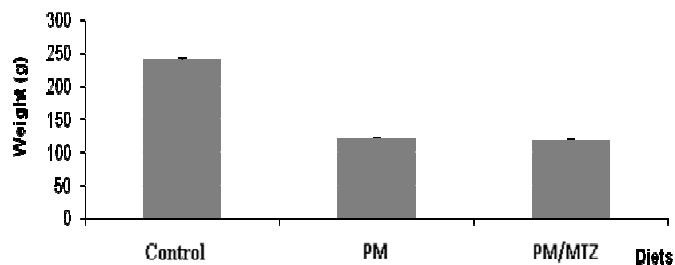

Figure 1. Weight loss in different situation of diets. Each point represents the Mean ± Standard Error (n= 6 rats/group). Control group rats were fed a conventional diet. Protein Malnutrition (PM) group rats fed maize diet (10 g/rat/day). Protein Malnutrition and Metronidazole (PM/MTZ) group rats fed maize diet (10 g/rat/day) with 500 units/mL of metronidazole in their drinking water.

During the experimental feeding period, the control group (n= 6) had free access to the standard diet (UAR, Villemoisson-sur- Orge, France) and water *ad libitum*. The malnourished (PM) group (n= 6) was fed only maize 10 g/rat/day (EPE Group Avicole de l'Ouest, Mostaganem, Algeria), the malnourished group treated with metronidazole (PM/MTZ) was fed only maize 10 g/rat/day (EPE Group Avicole de l'Ouest, Mostaganem, Algeria) and they received in their drinking water 500 units/mL of metronidazole (J. B. Chemical and Pharmaceuticals Ltd. India). The drinking water pot was renewed every 24 h.

Food intake was measured daily at 17 h. After 10 days of food depletion, the rats were weighed at the end of each treatment and killed by cervical dislocation.

Testing for bacterial translocation

The mesenteric lymph nodes (MLN) complex and liver were excised and quantitatively cultured to assess the effect of protein malnutrition, protein malnutrition associated with metronidazole on bacterial translocation.

Using sterile procedures, the chest and abdominal cavities were reflected with sterile forceps and the exposed viscera were swabbed with a sterile, cotton-topped applicator stick, which was placed in a tube of brain-heart infusion. The tube was incubated aerobically at 37°C for 24 h to test for bacterial contamination of the viscera. The MLNs and liver were removed, and all organs were weighed separately. The MLN complex was placed in a sterile grinding tube and homogenized with 9 vol of brain-heart infusion using sterile ground-glass stoppers (Heimo et al., 2001). To determine bacterial concentrations of homogenates, each organ was diluted in decimal steps up to 1:10^5 in sterile solution.

After manual grinding, 1 ml of the homogenate was transferred into a tube containing 9 ml of physiologic serum; from this dilution 100 µl aliquots were plated on DRIGALSKI agar for enterobacteria culture (Sanofi, Diagnostic Pasteur, France). A further 1 ml aliquot was used to perform serial dilutions, portions of which were also plated on the three different plates as described before. Liver was analyzed in the same way as the MLN.

All agar plates for aerobic culture were incubated at 37°C under aerobic conditions for 1 day and then interpreted by an unbiased microbiologist. The gram-negative enteric was identified using the API 20 E system (Analytab Products, Plainview, New York).

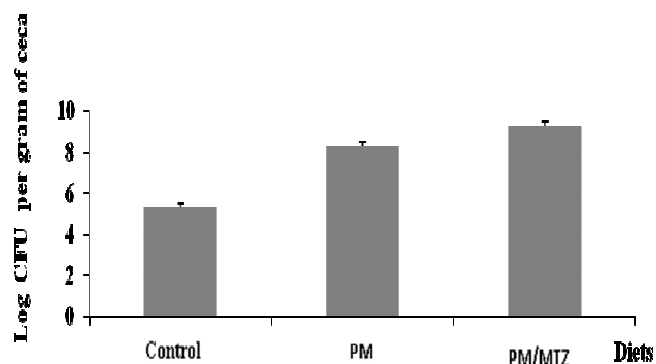Quantitative culture results were determined by the number of

Figure 2. Mean log 10 population level of enterobacteria per gram of ceca of rats in different situation of diet. Each point represents the Mean ± Standard Error (n= 6 rats/group). Control group rats were fed a conventional diet. Protein Malnutrition (PM) group rats fed maize diet (10 g/rat/day). Protein Malnutrition and Metronidazole (PM/MTZ) group rats fed maize diet (10 g/rat/day) with 500 units/mL of metronidazole in their drinking water.

Colony Forming Units per gram calculated from the dilutions of organ homogenates and positive tissue cultures. We did not study obligate anaerobic because these organisms are rare members of the intestinal flora of rodents early in life (Raibaud, 1988) and because they have a low tendency to translocate to extra intestinal sites (Stefen et al., 1988; Stefen and Berg, 1983).

Statistical analysis

Data are expressed as means ± standard error of the mean. The differences between the different groups were evaluated by chi square analysis with the Yates correction. Continuous data are expressed as mean ± SEM and analyzed with analysis of variance (ANOVA) and the Student unpaired t test. A P-value <0, 05 was considered significant.

RESULTS

The weight is a parameter which makes it possible to evaluate the total nutritional state of the animal and the effectiveness of the method suggested. The results obtained show that the body weight of the various groups of rats is variable from one groupto another (Figure 1). PM had a significant effect on body weight during the 10 days of the experimental period. Mean body weight of malnourished rats (117 ± 7 g) and malnourished rats treated with MTZ (120 ± 9 g) was lower than control group (239 ± 15 g), p<0.01. The loss of the body weight is probably due to the lack of essential nutrients like the

essential amino acids, the short chains fatty acids and vitamins of maize diet.

The enterobacteria counts of cecal flora are shown in Figure 2. The ceca enterobacteria overgrowth of malnourished rats is 8.306 ± 0.17 Log UFC/g of ceca. The
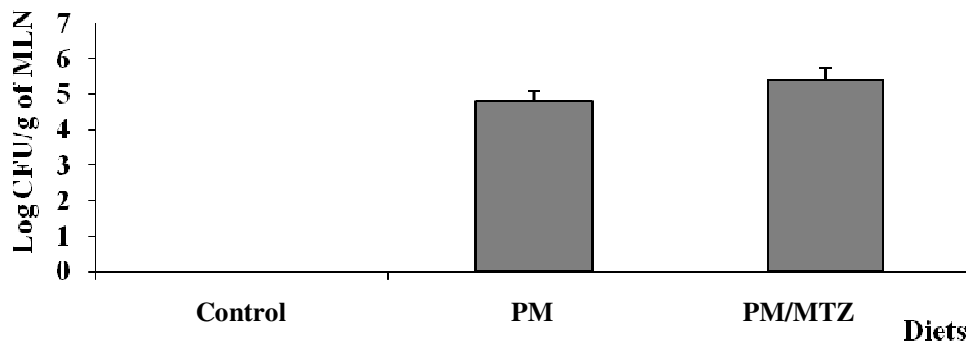

Figure 3. Translocation of enterobacteria to the MLN in different situation of diets. Each point represents the Mean ± Standard Error (n= 6 rats/group). Control group rats were fed a conventional diet. Protein Malnutrition (PM) group rats fed maize diet (10 g/rat/day). Protein Malnutrition and Metronidazole (PM/MTZ) group rats fed maize diet (10 g/rat/day) with 500 units/mL of metronidazole in their drinking water.

Figure 4. Translocation of enterobacteria to the liver in different situation of diets. Each point represents the Mean ± Standard Error (n= 6 rats/group). Control group rats were fed a conventional diet. Protein Malnutrition (PM) group rats fed maize diet (10 g/rat/day). Protein Malnutrition and Metronidazole (PM/MTZ) group rats fed maize diet (10 g/rat/day) with 500 units/mL of metronidazole in their drinking water.
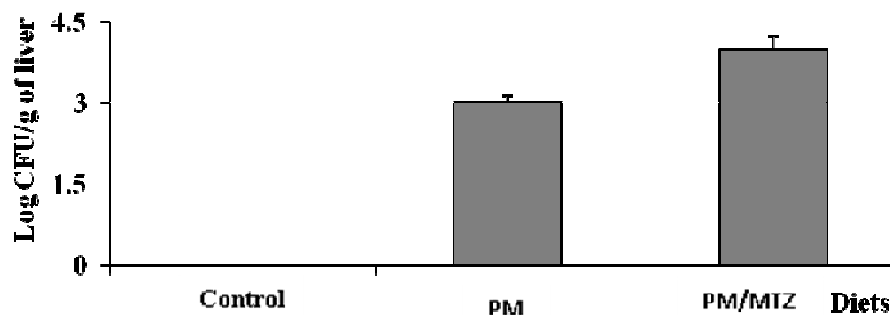
enterobacteria gram-negative counts of cecal flora were significantly higher in malnourished rats treated with MTZ than in control group, 9.25 ± 0.24 Log UFC/g of ceca versus 5.3 ± 0.17 Log UFC/g of cecal ($P < 0.01$). There were no significant differences between malnourished rats and malnourished rats treated with MTZ ($p > 0.05$). The effect of the MTZ in malnourished rats more disturbs the quantitative and qualitative balance of the bacterial flora of ceca.

Enterobacteria translocation was not seen in the control group. The incidence of Enterobacteria translocation in malnourished rats and malnourished rats treated with MTZ appears in the MLN were 4.84 ± 0.3 UFC/g of MLN and 5.22 ± 0.31 UFC/g of MLN, with respectively ($p <$

0.001; Figure 3). Although the number of translocating bacteria per gram of MLN was slightly increased by the administration of MTZ, there was no significant difference between malnourished rats and malnourished rats treated with MTZ. The incidence of enterobacteria translocation can be extend to liver with a frequency less than that noted in the MLN; 3.05 ± 0.13 UFC/g of liver in malnourished rats and 4 ± 0.23 UFC/g of liver in malnourished rats treated with MTZ (Figure 4).

DISCUSSION

Many different experimental models have been developed to determine the importance of bacterial translocation. In this study, the effect of protein malnutrition and protein malnutrition associated with metronidazole were evaluated on the body weight, the level of bacteria ceca population and translocation of enterobacteria to MLN, spleen and liver. Translocation of indigenous enterobacteria ciated with overgrowth of purely aerobic flora. Antibiotic therapy is known to alter microflora of the intestinal tract by the eradication or suppression of selected populations of bacteria while permitting resistant microbes to flourish (Virmeet et al., 2003).

In previous studies, we found that PM promote bacterial translocation, although the protein malnourished

from the intestinal tract to the MLN is associated with high population of these bacteria in the ceca. There is no evidence available as the route by which the indigenous bacteria cross the gut mucosa. The fact that bacterial translocation did not occur in the protein malnourished animals unless they were further challenged with endotoxin was puzzling, because the protein malnourished animals lost significant amounts of their body weight and sever mucosal atrophy (Katayama et al., 1997).

Katayama et al. (1997) found that protein malnutrition alone did not promote bacterial translocation, although the protein malnourished rats were more susceptible to endotoxin- induced bacterial translocation, and the magnitude of bacterial translocation and the mortality rate after endotoxin challenge were related directly to the degree of protein malnutrition. The study of jejuna aspirates of malnourished Australian aborigines and Guatemalan children show that they have significant intestinal bacterial overgrowth (Gracey and Stone, 1972). The indigenous microflora has an important control mechanism, helping to prevent bacterial overgrowth and to confine these microorganisms to the gut lumen. Disequilibrium of the intestinal ecosystem produced by immunosuppressor and antibiotics help to promote bacterial translocation to the mesenteric lymph nodes complex (Berg, 1980).

The major risk of antibiotic induced bacterial overgrowth is bacterial translocation and bacteraemia or septicaemia. Usually, bacterial overgrowth is resistant to the initial antibiotic (Levy, 2000). Protein deficiencies, in essential fatty acids, metal- enzymes, vitamin factors and oxidizing anti-elements decrease the immunizing functions. Recently, Rana and Bhardwaj (2008) have shown that small intestinal bacterial overgrowth (SIBO) is charac-terized by nutrient malabsorption associated with an excessive number of bacteria in the proximal small intestine. The pathology of this condition involves competition between bacteria and the human host for ingested nutriments. The intestinal flora ensures the maturation of the intestinal immune system represented by the Peyer's patches.

When overgrowth of bacteria occurs in the small bowel proximal to the distal ileum, symptoms of vitamin malabsorption, malnutrition, and weight loss may occur. However, in antibiotic therapy, most patients with small bowel bacterial overgrowth have aerobic and anaerobic overgrowth, and in others malabsorption has been asso-

associated with metronidazole were more susceptible to induced bacterial translocation and the incidence rate was higher than PM alone. The aerobic gram-negative bacilli translocate easily than anaerobic bacteria. Moreover, anaerobic bacteria act as a carpet on the mucosal surface, limiting colonization and overgrowth of other potentially invasive microbes. In fact, selective elimination of anaerobic bacteria facilitates intestinal overgrowth and the translocation of facultative bacteria.

Mucosal atrophy, particularly as a consequence of luminal nutrient deprivation, has been suggested as a predisposing factor for bacterial translocation. However, several experimental models of bacterial translocation could not convincingly demonstrate that morphological changes cause intestinal hyper permeability and/or bacterial translocation (Reiner and Heiko, 2003). Moreover, a situation of malnutrition associated or not with the metronidazole involves an increase in the population of the enterobacteria of ceca, although in a normal state their rate is much weaker compared to the number of strict anaerobic bacteria of the ceca flora. Thus, the collapse of the strict anaerobic total colony count armature can lead to an invasion of the enterobacteria through the intestinal mucosa probably more centered on the level of the segment ilea.

The passage of gram-bacteria of the intestinal light in the organization is at the origin of bacteremia which consequently depend on the immunizing statute of the subject. This translocation is perhaps increased by using immunosuppressor and/or by the antibiotic administration which, while destroying the anaerobic populations, will allow the overgrowth of enterobacteria of the intestinal flora (Wells et al., 1988; Berg et al., 1988). There are multiple possible routes by which an organism can translocate out of the gut to extra-intestinal sites; etrograde migration to the lungs, direct transmural migration across the intestinal wall, lymphatic migration via Peyer's patches, MLNs, the thoracic duct and the systemic circulation or via vascular channels to reach the portal system. There is substantial evidence that overgrowth in the upper intestine facilitates aspiration and retrograde migration of intestinal bacteria in severely stimulated by protein malnutrition. The majority of Peyer's patches are located in the small intestine, and our data indicate a relationship between bacterial population of ceca and translocation to the MLN.

Specific pathogen-free mice were treated orally with antibiotics to determine whether the resulting disruption of the normal flora ecology would allow certain gram-negative enteric bacteria to overpopulate bacterial ceca, thereby promoting the translocation of these bacteria from the gastrointestinal tract. Metronidazole treatment slightly increased the anaerobic bacterial levels. Penicillin or metronidazole treatment increased the enteric bacilli

populations in the ceca an average of 1,000-fold and promoting their translocation to the MLN (Berg, 1981). Many studies show that long-term therapy and high metronidazole concentrations could interfere with microbial pathogenicity, resulting in changes to host-bacterium relationships (Rodriguez et al., 1996).

Recent studies showed that the rate of gram negative enterobacteria, adhered to the ileum and with the ceca, in malnourished rats is less significant compared to that observed in rats, to which endotoxin are administered (Katayama et al., 1997). In this study, the impact of protein malnutrition in partnership with the administration of the metronidazole on the appearance of the ceca bacterial overgrowth promote translocation of the enterobacteria from the gastrointestinal tract to the MLN and liver.

In summary, the problem of protein malnutrition and the use of antibiotics to prevent infections and the phenomenon of bacterial translocation have received increasing attention in recent years, because the ability of bacteria to translocate across the mucosa barrier to MLN, and other extra intestinal sites like liver appears to be an essential step in malnutrition induced bacterial translocation. These microbes can cause further polymicrobial septic complications in different organs causing bacteremia and/or septicemia.

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