Effect of Metronidazole on the Pathogenicity of Resistant Bacteroides Strains in Gnotobiotic Mice

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Metronidazole is widely used to treat protozoan and fungal infections. As an antibacterial drug, it is used mainly against anaerobes. Among anaerobes, the Bacteroides fragilis group is the most relevant in terms of frequency of recovery and antimicrobial resistance patterns. The use of metronidazole and other antimicrobial drugs induces morphological changes in this bacterial group. The present study investigated in vivo if these morphological modifications were accompanied by changes in virulence patterns by using germfree mice experimentally challenged with metronidazole-resistant Bacteroides strains before and after exposure to metronidazole. It was observed that metronidazole-resistant strains were more virulent after contact with the drug, as demonstrated by anatomicopathologic data for spleen, liver, and small intestine samples. These results suggest that long-term therapy and high metronidazole concentrations could interfere with microbial pathogenicity, resulting in changes to host-bacterium relationships.

Materials and Methods

Bacterial strains. Two metronidazole-resistant strains identified with the API 20A kit (BioMérieux S.A., Marcy l’Etoile, France) as Bacteroides distasonis were used. One of them was isolated from a human intra-abdominal abscess (RH), and the other was isolated from the gastrointestinal tract of a healthy marmoset (Callithrix penicillata) (RM). Both of them were metronidazole-resistant (the MIC at which 90% of the isolates tested was 256 μg/ml for RH and 512 μg/ml for RM) according to the agar dilution test and were stored at −80°C in our culture collection. They were divided into four groups distinguished by maintenance in the presence of (RHmzol and RMmzol) or absence (RH and RM) of metronidazole. The strains were grown in 5 ml of brain heart infusion supplemented with hemin, menadione, and yeast extract (BHI-S), either containing or not containing 200 μg of metronidazole (lot no. 54H0407; Sigma Chemical Company, St. Louis, Mo.)/ml. The bacteria were grown for 24 h at 37°C in an anaerobic chamber (Forma Scientific Inc., Marietta, Ohio) containing an atmosphere of 85% N2, 10% H2, and 5% CO2.

Animals. Germfree NIH (Taconic, Germantown, N.Y.) 21-day-old mice were used in this study. The animals were housed in flexible plastic isolators (Standard Safety Company, Palatine, Ill.) and were handled according to established procedures (20). The animals were fed an autoclavable commercial diet for rodents (Navital, Curitiba, Brazil). Experiments with gnotobiotic mice were carried out in microisolators (UNO Roestvastaal B.V., Zevenaar, The Netherlands).

Experimental challenge. Thirty germfree mice were divided into five experimental groups of six animals. Each group was designated as follows, according to the associated bacterial strain: group 1, RHmzol; group 2, RH; group 3, RMmzol; group 4, RM; and group 5, control germfree. The groups were inoculated intragastrically with 0.1 ml of anaerobically grown (for 24 h) suspensions in BHI-S of RHmzol-RMmzol, RH, and RM which contained about 108 CFU/ml.

Microbial counts in gnotobiotic groups. Feces collected by anal stimulation from all mice every 2 h after the challenge were introduced into the anaerobic chamber, diluted 100-fold in regenerated sterile buffered saline (pH 7.4), and homogenized by hand. Serial 10-fold dilutions were obtained, and 0.1-ml amounts were plated onto BHI agar supplemented with hemin, menadione, and yeast extract (BHI agar-S). The petri dishes were incubated for 48 h in the anaerobic chamber at 37°C, after which colonies were counted. The monoxenic (groups 1, 2, 3, and 4) or germfree (group 5) status of the animals was regularly tested, and metronidazole resistance was reevaluated after recovering the Bacteroides strains from mouse feces (BHI agar-S containing 200 μg of metronidazole/ml).

Histopathologic evaluation. All experimental and control animals were sacrificed by ether inhalation and necropsied after 14 days of infection. Spleen, liver, and small intestine samples were excised and evaluated macroscopically. Small intestine samples were identified as duodenum, proximal jejunum, distal jeju-

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num, and ileum according to established procedures (7). Fragments of spleen, liver, and small intestine were fixed in formaldehyde–phosphate-buffered saline (1:10, pH 7.2) and dehydrated in alcoholic solution by using an automated tissue processor (Titertek Autotechnicon, Technicon tissue processor no. 2A; The Technicon Company, Chauncey, N.Y.). The fragments were embedded in paraffin, and 4-μm cross sections were obtained with a microtome (no. 820; Spencer, Buffalo, N.Y.). The slides were stained with hematoxylin and eosin, coded, and examined by a single pathologist, who was unaware of the experimental conditions for each group.

**Statistical analysis.** Some data were evaluated by analysis of variance. Statistical analysis was performed with EPISTAT software (T. L. Gustafson, Round Rock, Tex.), with the level of significance set at a value of $P < 0.05$.

**RESULTS**

We observed that during and after metronidazole exposure, the samples (RHmzol and RMmzol) showed altered morphology with filamentous cell formation (Fig. 1).

Figure 2 shows that the Bacteroides strains became established in the digestive tracts of all experimental gnotobiotic groups and that the number of CFU was about $10^9$/g of feces.

The macroscopic examination of livers from animals infected with all the Bacteroides strains did not show any apparent alteration. Spleens from mice infected with the RHmzol strain proved to be seriously damaged. Significant atrophy was observed when they were compared with spleens from animals infected with RMmzol, RH, and RM and from the control group (Fig. 3). Analysis of variance of the spleen areas showed that this atrophy was statistically significant ($P < 0.05$). Macroscopic examination of the small intestine showed hemorrhagic areas in the duodenum and proximal jejunum.

Histopathologic examination of spleen, liver, and intestine samples confirmed the macroscopic data, showing significant lesions in the animals infected with the RHmzol and RMmzol strains. Lesions or alterations were not observed in the group infected with the RH and RM strains or in the control group. The intestinal lesions were markedly expressed as altered villi with erythrocytes in the mucous tissue and congested blood vessels (Fig. 4). These lesions, however, were more evident in the animals infected with the RHmzol strain. Microscopically, livers from animals infected with RHmzol and RMmzol showed some hemorrhagic areas with congested blood vessels. The same was not observed in the animals infected with the RH and RM strains or in the control group. In spleens from mice infected with the RHmzol strain, a reduction of the lymphoid component and red pulp was observed when those spleens were compared with spleens from animals infected with RMmzol, RH, and RM and from the control group (Fig. 5).

**DISCUSSION**

There is a delicate balance in the intestine among the number of microbes and their virulence characteristics and the immune status of the host. Infection occurs whenever a microbe, whether by exposing the host to a larger infective dose...
or by increasing its own virulence characteristics, manages to disrupt the balance in its favor. Many of the bacteria in the gastrointestinal ecosystem, especially gram-negative rods, are opportunistic pathogens and are capable of eliciting injurious, proinflammatory responses if viable bacteria or soluble cell surface components or metabolic products interact with cells of the immune system (12). This indigenous microbiota may be pathogenic during an eventual disequilibrium of the digestive ecosystem or when these microorganisms are introduced into sterile areas of the body (3, 25). This disequilibrium may be due to immunosuppression, infectious or noninfectious diseases, or long-term therapy with antimicrobial agents.

Virulence factors may vary among individuals in the same microbial population. Thus, any agent that can interfere with their genetic profiles may influence the initial virulence properties of this population (16). In the B. fragilis group, the virulence has mainly been related to capsular polysaccharide, lipopolysaccharide, hydrolytic enzymes, and exotoxin (22). Various studies have indicated that short-chain fatty acids are also important virulence factors which affect the host defense mechanisms. Butyric acid, in particular, inhibits T-cell proliferation induced by different mitogenic stimuli (13).

Since the early 1940s many authors have observed morphological changes in various microbial species in the presence of different antimicrobial agents. In almost all cases these alterations are related to differences in the virulence patterns of such microorganisms (8, 11, 15, 23). In various studies, elongated cells of B. fragilis were observed in the presence of metronidazole, as was the case with B. distasonis strains during and after metronidazole exposure in this study. Elongation appears to result from the inhibition of autolytic enzymes that initiate separation. Nevertheless, the extent and consequences of these aberrant forms for the virulence properties of the B. fragilis group are unclear.

It has been previously demonstrated that antimicrobial agents are capable of interfering with the adhesiveness and enzyme and toxin production of different microbial groups. The β-lactams, quinolones, tetracyclines, glycopeptides, macrolides, lincosamides, and nitroimidazoles may stimulate adhesiveness to both epithelial and intestinal cells and the production of hemolysins, penicillinases, enterotoxins, other toxins, and enzymes by different species, such as Staphylococcus aureus, Streptococcus pyogenes, Streptococcus pneumoniae, Escherichia coli, Vibrio cholerae, Clostridium difficile, and B. fragilis (8, 14, 15, 17, 21, 26, 27). The enhancement of surface anionicity and hydrophobicity of Bacteroides by metronidazole, as an example, may allow the bacteria to approach negatively charged surfaces of animal cells more efficiently.

In this study, the metronidazole-resistant strains showed differences in host-microbial interaction after exposure to metronidazole. RHmzol and RMmzol were more pathogenic, as shown by the histopathologic data. However, RHmzol was more virulent and had an unexpected and strong effect on the spleen in particular, which atrophied during the infection. It is well established that red pulp may function as a blood reserve. The atrophy of this spleen component may be related to hemorrhagic processes like that observed in the intestines of animals challenged with metronidazole-resistant strains maintained in the presence of this drug. On the other hand, white pulp is

![FIG. 3. Mean areas of the spleens from germfree (control) and gnotobiotic mice infected for 14 days with B. distasonis strains previously treated (RHmzol and RMmzol) or not (RH and RM) with metronidazole. Different letters above bars indicate that results were significantly different (P < 0.05).](image)

![FIG. 4. Histological examination of small intestine mucosae from gnotobiotic mice infected for 14 days with resistant B. distasonis strains not treated (RH [A]) or previously treated (RHmzol [B]) with metronidazole. White arrows show the appearance of blood vessels (normal [A] and congested [B]). Black arrows (B) indicate mucosal hyperemia, showing blood cells at the basal mucosa of intestine from an animal challenged with RHmzol. Interestingly, strain RMmzol behaved very similarly to RHmzol. Hematoxylin and eosin were applied. Original magnification, ×240.](image)
comprised of lymphnodes and should grow during the acute immune response. However, during immunosuppression, these lymphnodes generally undergo degeneration by cellular apoptosis, which causes a reduction in the size of most lymphoid organs (mainly the spleen) (2). This pattern also establishes the interference of anaerobic bacteria with the host’s immune response, acting as lymphocyte suppressor agents (6).

The present results suggest that antibiotic resistance in microorganisms leads to an increase in morbidity and mortality not only by increasing the risk of inappropriate therapy but also by simultaneously increasing the virulence of such resistant microorganisms. Further prospective clinical, pathologi- cal, and immunological investigations are needed to elucidate the level of interference of metronidazole and other antimicrobial agents, mainly in the remaining susceptible and indigenous microorganisms, as established by the American Society for Microbiology (1) and by the European Societies of Health (18).

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