ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1978, p. 115-120

Vol. 13, No. 1
Printed in U.S.A.

Effect of Metronidazole on the Intestinal Microflora of the American Cockroach, *Periplaneta americana L.*

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Received for publication 8 November 1977

The effect of feeding to cockroaches the antimicrobial agent metronidazole, which acts specifically against anaerobes, was assessed by light and scanning electron microscopy, bacteriological examination, and methane formation. Types of organisms and total numbers were greatly reduced from controls. The health of mature adults was unaffected, but stunting occurred in subadult animals maintained on the antibiotic from hatching. The intracellular bacteria of the fat body were not affected by the drug. The results are discussed with respect to a proposed microbe-host extracellular symbiosis.

Studies of the alimentary canal of the cockroach indicate the presence of an elaborate hindgut microflora (7). Many of the attached microbes and unusual morphological forms have been refractory to isolation. As an alternative to the isolation and characterization of all the anaerobes possible, we have approached the problem of interactions of the hindgut flora with the animal via the possibilities of transport of microbial products across the hindgut wall (J. W. Bracke and A. J. Markovetz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, I169, p. 180), and in this study by the specific elimination of the anaerobic hindgut flora to ascertain if such treatment affects the host.

Metronidazole acts specifically against anaerobic microorganisms by being reduced by a phosphoroclastic reaction (6) with the reduced form of the drug presumably causing the formation of a less stable deoxyribonucleic acid (5). Since methane formation by the cockroach is due to the activities of anaerobic bacteria in the hindgut, its formation was used as an assay for determining the effect of metronidazole on hindgut flora. Elimination of the mycetomes, the cells harboring the intracellular bacteria in the fat bodies of cockroaches, results in stunting and impaired reproduction of the host (3), and it was therefore important to determine that physiological effects of metronidazole were not the result of the elimination of the mycetomes as well as of the gut flora.

MATERIALS AND METHODS

Animals. A colony of *Periplaneta americana L.* was maintained in the laboratory in a large aquarium with San-I-Cel laboratory animal bedding (Paxton Processing Co., Paxton, Ill.) on Teklad Mouse and Rat Diet (4% fat; Mogul Corp., Winfield, Iowa) and water ad libitum. The chow and bedding were steam sterilized before use.

Media. Numerous liquid and agar plate media, primarily chopped meat and peptone-yeast extract-glucose supplemented with menadione and hemin, from the Virginia Polytechnic Institute Manual (9) were prepared aerobically and reduced in an anaerobic chamber immediately after sterilization. All bacterial cultures were grown in an anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.).

Antimicrobial agent. Metronidazole (Flagyl, G. D. Searle and Co., Chicago) was incorporated into the drinking supply at levels of 200 and 1,000 μg/ml. Antimicrobial susceptibility was determined by the antimicrobial disk method (12) and the agar dilution technique (2) by using twofold serial dilutions to 2,048 μg/ml.

Feeding experiments. Mature adult cockroaches were placed into sterile containers with sterile food and water containing the antibiotic. Newly hatched animals were taken from a brood of 26 and split into 2 unsexed groups. The control group was fed a measured amount of sterile animal chow and water. The test group was fed the same quantity of food, but the drinking water contained metronidazole.

Methane determination. Methane production was determined by placing animals in 22-ml vials sealed with black rubber stoppers. After a period of from 1 to 2 h, the gas in the vials was sampled by inserting a gas-tight syringe needle through the stopper. Samples were injected into a Varian Aerograph Series 2700 gas chromatograph, equipped with a Spherocarb 80/100 mesh column (6 feet by 1/8 inch [ca. 1.9 m by 0.32 cm]) at 60°C. Animals appeared to suffer no ill effects from the confinement.

Microscopic examination. Colons were dissected out, slit lengthwise, and vortexed in insect Ringer solution (8) to remove the lumen contents. They were then fixed by a modified osmium tetroxide-

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thiocarbohydrazide binding technique (10), dehydrated, and critical-point dried before examination with a Kent Cambridge Stereoscan S4 scanning electron microscope. Light microscopy of the intracellular bacteria was performed on thin leaflets of fat body in Ringer solution in wet mount and Gram-stained preparations.

RESULTS

Adult cockroaches fed metronidazole did not change in appearance or behavior over a period of one year. Their intestinal microflora, however, was drastically reduced within one month of the start of the antibiotic experiment. The

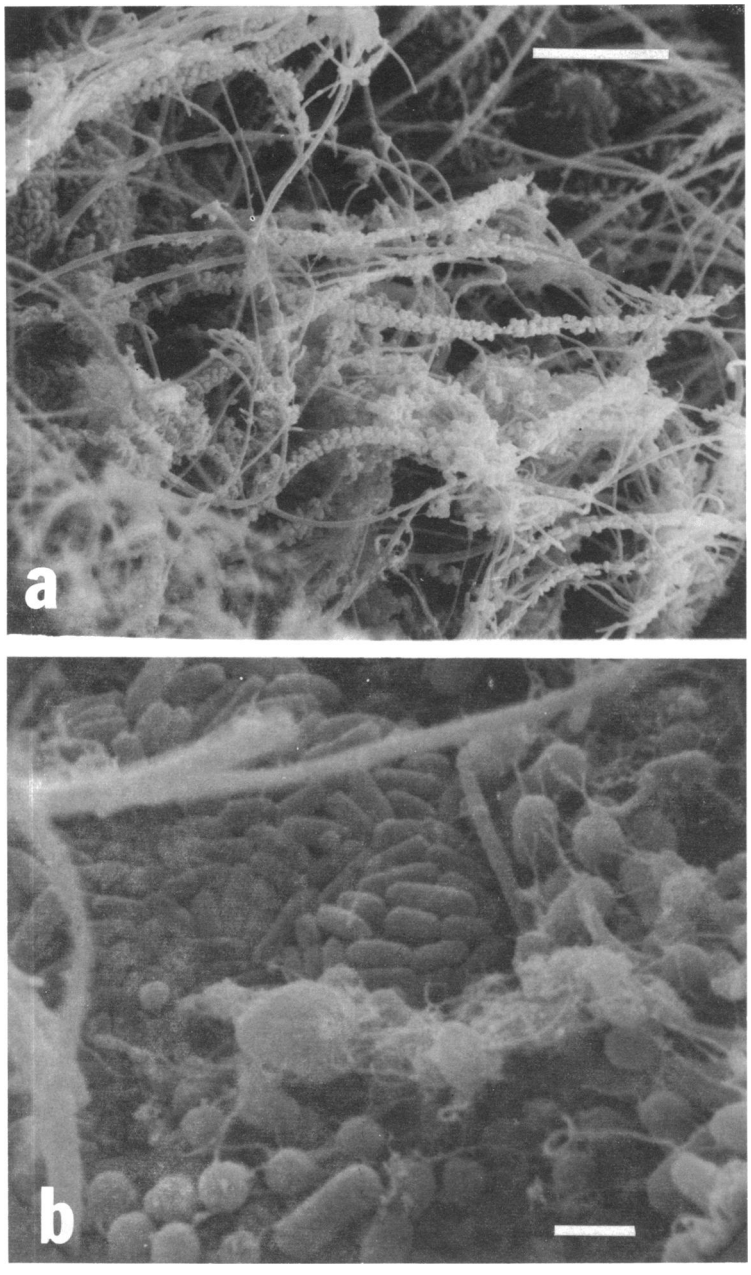

FIG. 1. *Scanning electron micrographs of colon microflora from P. americana (2). (a) Aerial microflora; magnification bar represents 1 μm. (b) Posterior region of the colon; magnification bar represents 1 μm.*

effect of either amount of metronidazole (i.e., 200 or 1,000 μg/ml) was judged the same, and the lower amount was routinely used to minimize possible toxic effects to the host.

Methane formation by adult control cockroaches, with animals weighing from 705 to 997 mg, varied from 1.5 to 11.5 μl/h (mean = 4.4 μl/h), whereas that of the subadult controls weighing from 195 to 370 mg ranged from 1.4 to 6.2 μl/h (mean = 2.8/h). No methane was detected in any animals, adult or subadult, given metronidazole for over 30 days. The sensitivity of the assay would have allowed detection of less than 0.1 μl/h.

Figures 1a and b show the upper and lower colon microflora, respectively, in control ani-

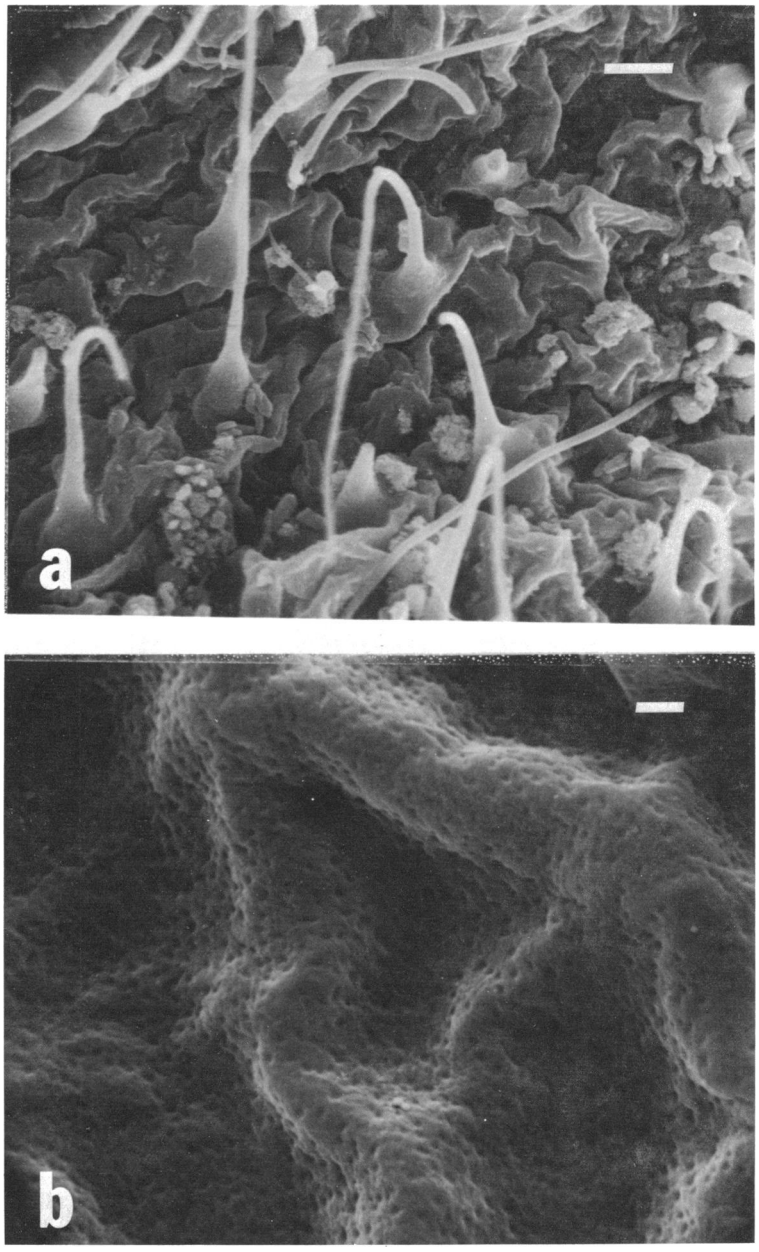

FIG. 2. *Scanning electron micrographs of the colon of metronidazole-treated P. americana. (a) Barren spines; magnification bar represents 1 μm. (b) Porous colon cuticle. Magnification bar represents 1 μm.*

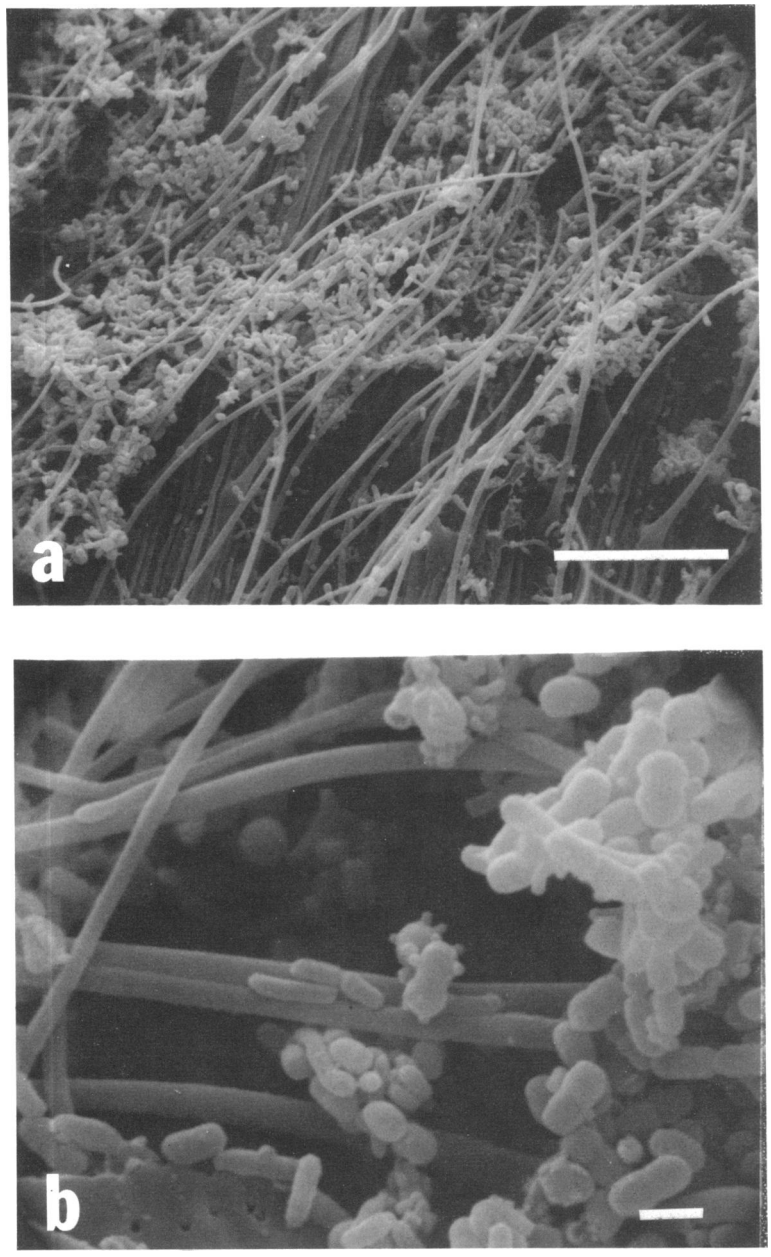

FIG. 3. *Scanning electron micrographs of the colon of metronidazole-treated P. americana. (a) Magnification bar represents 10 μm. (b) Prosthecate organisms; magnification bar represents 1 μm.*

mals examined by scanning electron microscopy. The corresponding regions of metronidazole-treated cockroaches are seen in Fig. 2a and b. Most of the microflora had been eliminated. Occasional patches of colonization could still be located if the colon was examined in detail (Fig. 3a and b). The morphology of the residual microflora in the colonized patches was dominated by a chaining coccus. An unusual form was also observed in low numbers that had not previously been observed in extensive morphological studies of untreated cockroaches. Cultures of the colon revealed only one isolable obligate anaerobic bacterial species, *Peptostreptococcus productus*. This organism was found to be resistant to levels of metronidazole in excess of 1,000 μg/ml regardless of the susceptibility assay employed. The

same species, when isolated from control animals, was susceptible to levels of metronidazole below 256 μg/ml.

Subadult cockroaches fed the antibiotic from hatching showed retarded weight gain (Table 1). These results indicate that the control group gained significantly more weight than the antibiotic-fed group. There was no significant difference in mortality between the two groups.

The intestine of the metronidazole-fed subadults was significantly less developed than that of the control littermates. The length of the midgut and hindgut of the metronidazole-fed animal was 10 mm, whereas that of the control was 17 mm. Gut walls were thinner, and the contents were translucent and gelatinous, as opposed to the semiliquid contents of the control animal. The control hindgut contained fragments of exoskeleton (common in cockroaches that eat shed exoskeletons), whereas that of the experimental animal did not. Light microscopic examination of the hindgut lumen contents of the metronidazole subadults revealed only the gram-positive chaining coccus seen in adults and lower numbers of gram-positive rods with blunt ends. Scanning electron microscopic examination of the hindgut of the metronidazole-fed subadult revealed large numbers of the chaining coccus apparently attached to the wall of the gut (Fig. 4). These observations were the same for numerous animals examined over a period of one year.

When the fat bodies of subadults fed metronidazole since birth and adults given the antibiotic were examined, no differences were detected between their mycetomes and those of controls. The cells were the same size and all were packed with large gram-variable rods, many of which were apparently dividing.

TABLE 1. *Differences in weight gain of subadult cockroaches exposed to metronidazole from hatching*

Age (days)	Control		Metronidazole		Wt. of metronidazole animals as % of control
	No. of animals	Avg wt (mg/animal)	No. of animals	Avg wt (mg/animal)	
0	13	19.2	13	19.2	100
40	13	40	13	28.4	71
107	12	167	11	137	83
164	11	373	11	300	80

DISCUSSION

The results indicate that metronidazole, administered orally to the cockroach, greatly reduces the extent of microbial colonization of the colon. Because of the suggested mode of action of metronidazole and the observed reduction in methane production by treated animals, the microflora appears to consist predominantly of anaerobes. However, at the concentrations tested, the antimicrobial agent does not completely eradicate the colon microflora. It is interesting that the most obvious remain-

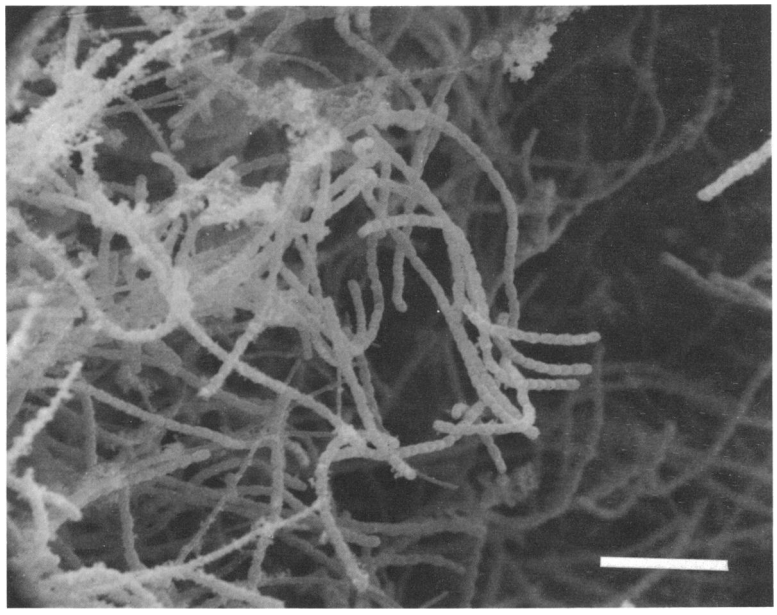

FIG. 4. *Scanning electron micrograph of colon of 164-day-old nymph, raised since hatching on metronidazole. Predominant microorganism is a chaining coccus. Magnification bar represents 10 μm.*

ing morphological form and the only isolable anaerobe in treated insects is a chaining coccus.

Although other studies have reported a tendency for metronidazole resistance in the anaerobic gram-positive cocci (4, 11), the high level of resistance in the current isolate of *P. productus* is unprecedented. In addition, *P. productus* is part of a group of anaerobic cocci that collectively represent approximately 24% of the clinical specimens positive for anaerobes (13). It is also part of the normal and diseased vaginal microflora (1, 14). Since metronidazole is the drug of choice for several genital protozoan infections and is used in some bacterial infections (6), it is reasonable to speculate that the number of clinically isolated, metronidazole-resistant strains of *P. productus* may increase if the drug is used indiscriminately.

The unusual morphological form observed in Fig. 3b was thought to be an artifact of the drug treatment. However, it has since been observed in cockroaches not exposed to metronidazole. It appears to be present in very low numbers and was not normally observed until the antibiotic reduced the amount of interfering microbial biomass. Its presence in the treated animals suggests that it is either another resistant anaerobe or a facultative species. A very similar organism has been observed by both scanning and transmission electron microscopy in the hindgut of the Dipteran larvae, *Tipula abdominalis* (M. J. Klug, personal communication).

Preliminary experiments with *Eublaberus posticus*, a cockroach distantly related to *P. americana*, indicate the same pattern when the animals are given metronidazole, i.e., loss of methane production, drastically reduced intestinal flora, retarded growth, and no loss of mycetomes (unpublished data). It appears that the drug directly interferes with an extracellular symbiotic relationship between the anaerobic colon bacteria and the host animal. This interpretation requires caution, however, since the experiments need to be continued through multiple generations to assess whether there is any direct toxic effect of the metronidazole on the cockroach tissues that could account for the observed retardation of growth.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant GM-19809 from the National Institute of General Medical Sciences and by grant AI-13990 from the National Institute of Allergy and Infectious Diseases.

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