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In vivo activity of nifurzide and nifuroxazide in intestinal bacteria in man and gnotobiotic mice

FLORENCE LEONARD, A. ANDREMONT & C. TANCREDE *Service de Microbiologie Médicale, Institut Gustave-Roussy, Rue Camille Desmoulins, 94800 Villejuif, France*

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Although Gram-negative enteropathogenic bacteria are the target strains of nifurzide and nifuroxazide treatments, neither drug affected faecal counts of *in vitro*-susceptible Enterobacteriaceae in healthy volunteers. This absence of activity was shown to be due to the poor solubility of the drugs tested. Therefore, effect of high doses of nifurzide was investigated in gnotobiotic mice. Activity against *in vitro* susceptible enteropathogens was then observed. Normal bacterial cells were replaced in the faeces by elongated, nonseptate and unflagellated mutants. Moreover, the resistance to colonization by enterotoxigenic *Escherichia coli* and *Shigella flexeri* of an anaerobic flora of human origin was sharply decreased.

Bactericidal and bacteriostatic activities produced by nitrofurans have been known since 1944 (Dodd & Stillman 1944). Nifuroxazide and nifurzide are synthetic members of this group of antimicrobial agents and their intestinal absorption has never been reported. They are used in Europe, Africa, the Middle East and the Indian Ocean in the treatment of infectious diarrhoea. *In vitro* susceptibility tests of these drugs against several bacterial species enteropathogenic for humans have shown that their minimum inhibitory concentrations (MICs) are low (Delsarte *et al.* 1981; Vanhoof *et al.* 1981). Oral administration of nifuroxazide is reported to cause no disturbance of the aerobic Gram-negative intestinal microflora and no increase in the plasmid-mediated antibiotic resistance of Enterobacteriaceae (Avril *et al.* 1981). The susceptibility to nifuroxazide of the clones isolated was not documented. Some data are available on the *in vitro* mechanisms of action of nifurzide and its binding to bacterial cells (Delsarte *et al.* 1981; Videz *et al.* 1981) but none have been published regarding the *in vivo* intestinal antibiotic activity of nifurzide or nifuroxazide. The controlled studies published concerning their clinical efficacy unfortunately lacked adequate microbiological data (Auzerie *et al.* 1982).

Gnotobiotic mice associated with a human intestinal flora have been shown to harbour in their gastrointestinal tract a microflora similar to that of the human donor (Raibaud *et al.* 1980; Hazenberg *et al.* 1981). This flora appears to be stable provided that the animals are kept isolated (Hazenberg *et al.* 1981). Intestinal antibiotic concentrations equivalent to those obtained in the intestine of human volunteers can be produced in these human flora-associated mice (Andremont *et al.* 1983). This model has been used to study the *in vivo* effect of antimicrobial agents on complex human microflora (Hazenberg *et al.* 1982; Hazenberg *et al.* 1983) and on resistance to intestinal colonization by exogenous micro-organisms (Andremont *et al.* 1983). In addition, mice are not susceptible to most of the bacterial strains which are enteropathogenic for humans. Intestinal colonization lasting several weeks was obtained with *Shigella flexneri* and *Vibrio cholerae* without any clinical symptoms, thus allowing microbial antagonisms to be observed for long periods (Freter 1962; Sasaki *et al.* 1970; Ducluzeau *et al.* 1977).

For these reasons we decided to examine (1) the effect of nifurzide and nifuroxazide on the endogenous Enterobacteriaceae of healthy

volunteers taking the daily doses of these drugs usually recommended and (2) the effect of high doses of nifurzide on a complex human microflora and on several Gram-negative enteropathogenic bacteria introduced in the intestine of gnotobiotic mice.

Materials and Methods

HUMAN VOLUNTEERS

Eighteen healthy, fully-informed adult volunteers were included in the study. None had taken antibiotics for at least one month before the study. Nifurzide was administered in 150 or 300 mg doses and nifuroxazide in 200 mg doses. Whatever the dose, it was given three times daily for 5 d. Six subjects were randomly assigned to each of the three regimens. Freshly passed faecal samples were obtained before, once a day during, and one week after treatment.

GNOTOBIOTIC MICE

Adult germ-free C3H mice (Centre de Sélection des animaux de laboratoire, Orléans, France) were maintained in plastic Trexler type isolators. They were fed a locally prepared diet (Andremont *et al.* 1983) sterilized by gamma irradiation. Autoclaved drinking water at pH 3 was given *ad libitum*. When necessary nifurzide was mechanically mixed with the components of the diet to a concentration of 1% before pelleting. Nifurzide activity was not modified by this procedure or by the irradiation (data not shown).

Half the mice received a complex human faecal flora by intragastric and intrarectal inoculation with a dilution of the original flora, as previously described (Andremont *et al.* 1983). To facilitate the counts of the challenge strains all Enterobacteriaceae has been removed from the complex human faecal flora used by microbial modification of the intestinal tract of the donor with erythromycin (Andremont *et al.* 1983). Earlier, precise analysis of the resulting flora in mice had shown that total bacterial counts, anaerobic composition and resistance to colonization by exogenous bacteria were not substantially modified by this procedure (Andremont *et al.* 1983). No antibiotic activity was detected in the faeces of the recipient mice.

All mice were challenged intragastrically, as already described (Andremont *et al.* 1983), with 1 ml of a culture containing 10^8 cfu/ml of one of the following strains: *V. cholerae* 569B, enterotoxigenic *Escherichia coli* H10407 producing heat-labile and heat stable enterotoxins (both obtained from Dr J.P. Craig, SUNY Brooklyn, New York), *Sh. flexneri* DKR 115, or *V. parahaemolyticus* J525C (both isolated by one of us from diarrhoeal patients). MICs of nifurzide for the challenge strains were 0·06, 8, 2 and 0·06 mg/l respectively. An interval of two weeks was allowed to elapse between the introduction of the faecal flora into the germ-free mice and their inoculation with the challenge strains. Treatment with nifurzide was begun two weeks after the mice had been challenged with the enteropathogens.

MEDIA

B′ agar (Raibaud *et al.* 1966) contained (g/l): yeast extract, 10; peptone, 15; tryptone, 10; tween 80, 1·0; agar, 10; pH 6·5. Aranki agar (Aranki *et al.* 1969) contained (g or ml/l): of trypticase soy, 40; yeast extract, 5; K_2HPO_4, 2·5; palladium chloride, 0·3; Na_2CO_3, 0·6; agar, 5; hemin, 0·001; D-glucose, 0·5; menadione, 0·005, cysteine hydrochloride, 0·5; decomplemented horse serum, 10; pH 7·5.

BACTERIAL COUNTS

Total bacterial counts were performed either on the solid Aranki medium in an anaerobic chamber (Aranki *et al.* 1969), or in B′ agar poured in 8 × 400 mm tubes (Andremont *et al.* 1983). In both cases the cultures were incubated for 7 d at 37°C. Anaerobic strains were classified into genera according to their shape and motility, Gram straining properties, shape and position of spores, respiratory type, catalase production, and glucose fermentation (Raibaud *et al.* 1966). Aerotolerant streptococci, Enterobacteriaceae, and vibrios were counted by plating respectively on Bile-Esculin agar, MacConkey agar and Thiosulphate Citrate Bile Sucrose agar (Difco). These cultures were incubated for 48 h at 37°C. Identification of the clones isolated was confirmed on the basis of the usual morphological and biochemical criteria.

SUSCEPTIBILITY ASSAYS

The MICs of nifurzide and nifuroxazide were determined by the agar dilution method (Washington & Sutter 1980) using a multiloop inoculating device which delivered 0·5 μl samples of bacterial culture to a plating agar containing twofold dilutions of nifurzide or nifuroxazide solubilized in acetone. Strains were cultured in brain heart infusion broth for 18 h at 37°C, and 10^{-5} dilutions of the cultures were used as inocula (10–100 cfu per spot). The MIC was the lowest concentration at which no visible colony appeared after 48 h at 37°C.

ASSAYS OF NIFURZIDE AND NIFUROXAZIDE

Freshly-passed human faeces and mouse faecal pellets were freeze-dried and stored at −20°C until assays were performed. Nifurzide and nifuroxazide activities were measured in the lyophilisates after they had been diluted in saline or acetone for nifurzide assays, or in saline, acetone or methanol for nifuroxazide assays. The influence of the binding of nifurzide to intestinal content and of its solubility on its antibiotic activity were tested *in vitro*. For this purpose the reference sample of nifurzide powder was mixed with autoclaved faeces (1 mg/g of faeces wet weight) of a human volunteer who had not undergone antibiotic treatment. The mixture was dissolved in saline, acetone, methanol or a mixture of 20% dimethylformamide and 80% acetone before being assayed for antibiotic activity. Nifurzide solutions free of faeces were used as controls. Antibiotic activity was determined by the agar diffusion assay (Sabath & Anhalt 1980) with Antibiotic Medium No. 2 (Difco) at pH 6·5. Control samples of nifurzide and nifuroxazide were diluted in acetone. The assay organisms were *Bacillus subtilis*, strain ATCC 6633, for nifurzide and *V. parahaemolyticus* strain IGR 19 for nifuroxazide. The respective sensitivities of the techniques were 1 μg/g and 10 μg/g of faeces. Confidence limits were ±10% for both assays (data not shown).

ELECTRON MICROSCOPY

A heavy bacterial suspension was made in saline from the colonies of the challenge strains isolated from the faeces of the mice either before or during the treatment with nifurzide. This suspension was negatively stained with uranyl acetate and examined with a Philips EM 300 microscope.

STATISTICAL ANALYSIS

Bacterial counts were converted into common logarithms. The log distribution of intestinal bacterial counts has been shown to be normal (Best 1970). Student's *t*-test was used for comparison of mean values.

Results

EFFECT OF NIFURZIDE AND NIFUROXAZIDE ON THE FAECAL ENTEROBACTERIACEAE OF THE HUMAN VOLUNTEERS

Mean faecal counts of Enterobacteriaceae were 6·85 $\log_{10}$ cfu/g before the beginning of the treatments. These counts were not significantly affected by any of the three dose regimens used, and they remained stable after the treatments had ended (Table 1).

Table 1. Counts of Enterobacteriaceae in the faeces of human volunteers before, during, and after nifurzide and nifuroxazide treatments*

	Treatment		
	Nifurzide (mg/d)		Nifuroxazide (mg/d)
Counts of Enterobacteriaceae	450 (n = 6)	900 (n = 6)	600 (n = 6)
Pre-treatment	6·80 ± 1·63	7·39 ± 0·66	6·07 ± 0·63
Per-treatment†	7·14 ± 1·32	7·11 ± 0·61	6·55 ± 1·36
Post-treatment	7·39 ± 0·92	6·95 ± 1·14	7·09 ± 1·66

* Values are means ±SEM $\log_{10}$ counts/g (n = number of volunteers).

† Counts from day 1–5 were pooled.

Table 2. Susceptibility to nifurzide and nifuroxazide of endogenous Enterobacteriaceae of human volunteers

Antibiotic	Phase of treatment	MIC_{50} (mg/ml)	MIC_{90} (mg/ml)
Nifurzide	Pre-treatment ($n = 70$)	4	8
	Per-treatment ($n = 86$)	4	8
Nifuroxazide	Pre-treatment ($n = 51$)	32	128
	Per-treatment ($n = 50$)	8	128

n, Number of strains tested.

Table 2 shows the susceptibility to nifurzide and nifuroxazide of 257 strains of endogenous Enterobacteriaceae: *E. coli* (201), *Klebsiella* spp. (27), *Enterobacter* spp. (27), *Proteus* spp. (1) and *Citrobacter* spp. (1) isolated from the 18 volunteers before or during their treatment. Nifurzide was more active than nifuroxazide, but no significant change in the MICs of either drug in endogenous Enterobacteriaceae was induced by the treatments.

EFFECTS OF HIGH DOSES OF NIFURZIDE ON ENTEROPATHOGENIC STRAINS AND ON HUMAN FAECAL FLORA IN GNOTOBIOTIC MICE

The four challenge strains were individually established in the intestine of germ-free mice at high concentrations (Table 3). These were stable with time (results not shown). When nifurzide was added to the diet, counts of *V. cholerae* 569B fell sharply. Counts of *Sh. flexneri* DKR 115 and *E. coli* H10407 changed only slightly but significantly (8·48 *vs* 9·49 and 8·51 *vs* 9·53, respectively; $P < 0{\cdot}01$ in both cases). Counts of *V. parahaemolyticus* J525C fell after treatment for 5 d but subsequently rose and stabilized at a concentration slightly but significantly lower than before the beginning of the treatment (8·28 *vs* 9·12 $\log_{10}$ cfu/g of faeces; $P < 0{\cdot}01$) (Table 3).

The colonies of the challenge strains obtained on agar media during the treatment were small and irregular. Electron microscopic examination of these colonies showed elongated, unsepted bacteria with no flagella (Fig. 1).

A high degree of colonization by challenge organisms was inhibited by prior establishment of complex human faecal flora in mice. *Vibrio cholerae* 569B and *V. parahaemolyticus* J525C were completely eliminated from the intestine within 48 h of the challenge. *Escherichia coli* H10407 and *Sh. flexneri* DKR 115 were still present in the faeces two weeks after the challenge but were confined to the subdominant flora (Table 3). When nifurzide was added to the diet, counts of *Sh. flexneri* DKR 115 and *E. coli* H10407 rose over 8·00 $\log_{10}$ cfu/g of faeces 10 d after the beginning of the treatment. These

Table 3. Faecal counts* of challenge strains in gnotobiotic mice before and during nifurzide treatment

	Mono associated mice†			Human flora-associated mice†		
		Per-treatment			Per-treatment	
Challenge strain	Pre-treatment‡	Day 5§	Day 10§	Pre-treatment‡	Day 5§	Day 10§
Escherichia coli H10407	9·53 ± 0·25	8·82 ± 0·70	8·51 ± 0·53	6·62 ± 0·34	7·85 ± 1·20	8·45 ± 0·63
Shigella flexneri DKR115	9·49 ± 0·22	8·34 ± 0·35	8·48 ± 0·43	3·94 ± 1·00	7·42 ± 0·31	8·19 ± 0·31
Vibrio cholerae 569B	8·95 ± 0·43	4·17 ± 1·50	3·77 ± 1·17	2·00	ND	ND
V. parahaemolyticus J525C	9·12 ± 0·31	5·25 ± 0·63	8·28 ± 0·52	2·00	ND	ND

* Values are means ± SEM $\log_{10}$ cfu/g of faeces.
† Six mice were inoculated with each challenge strain.
‡ Two weeks after challenge.
§ After the beginning of treatment.
ND, not done.

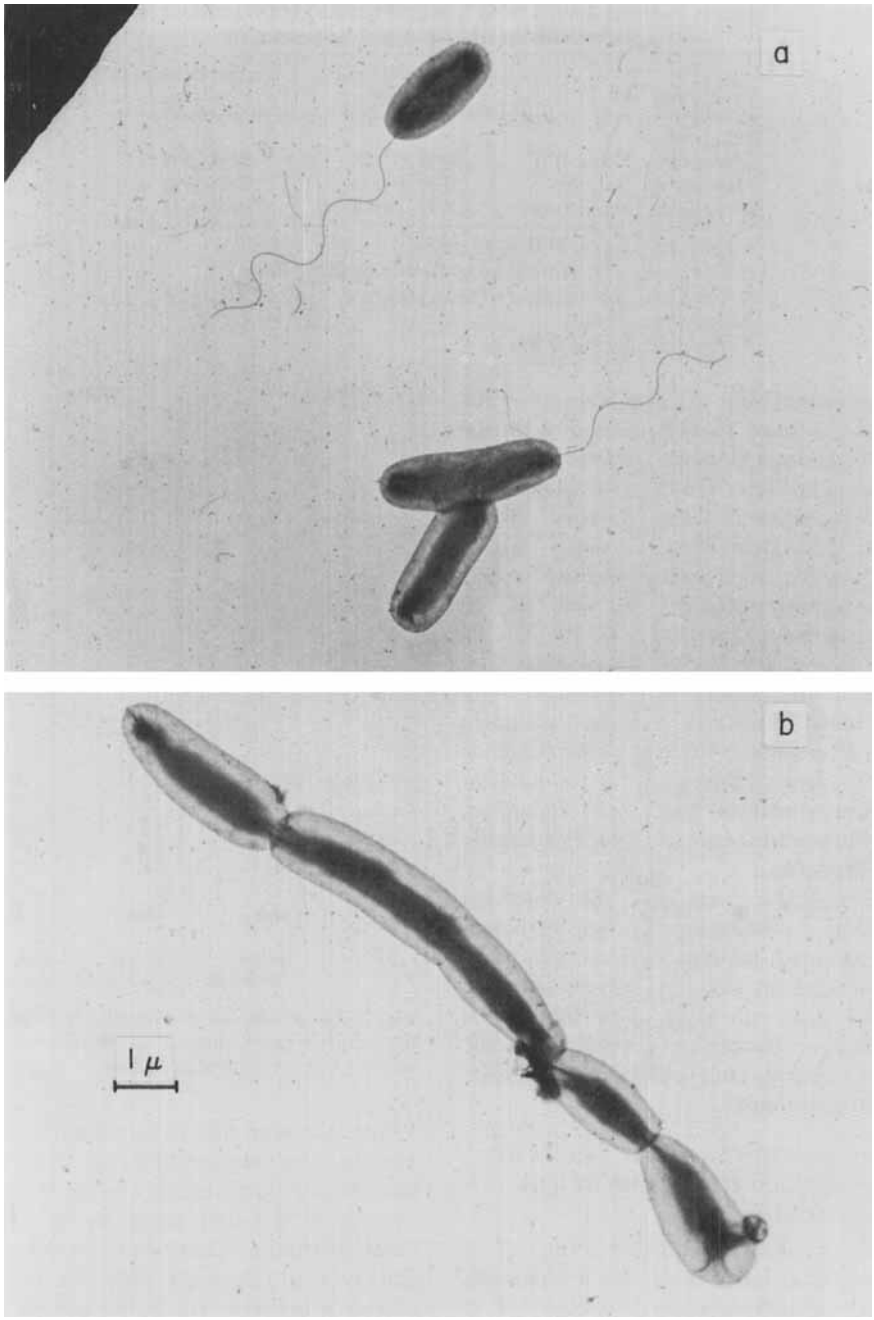

Fig. 1. Electron photomicrographs of cells of *Vibrio parahaemolyticus* J525C isolated from control (a) or nifurzide-treated (b) gnotobiotic mice.

Table 4. Bacterial counts* in the faeces of human flora-associated gnotobiotic mice before and during nifurzide treatment

Bacteria	Before treatment† ($n = 8$)§	During treatment‡ ($n = 6$)§
Total counts		
Anaerobic chamber¶	10·48 ± 0·26	10·08 ± 0·24
8 × 400 mm tubes¶	9·54 ± 0·44	10·03 ± 0·50
Aerotolerant streptococci	6·88 ± 0·71	9·59 ± 0·50

* Data are mean ± SEM log counts/g.
† Two weeks after association of the mice with the flora.
‡ Ten d after the beginning of the treatment.
§ *n*, number of mice.
¶ See Materials and Methods.

counts were equivalent to those observed in the faeces of the mice monoassociated with the same strains during nifurzide treatment.

As shown in Table 4, counts of aerotolerant streptococci increased sharply during the treatment with nifurzide (6·88 *vs* 9·59 $\log_{10}$ cfu/g of faeces; $P < 0{\cdot}01$). Total anaerobic counts as performed in the anaerobic chamber were reduced by nifurzide treatment (10·08 *vs* 10·48 $\log_{10}$ cfu/g of faeces; $P < 0{\cdot}05$). As expected, total counts measured before this treatment were higher when measured in the anaerobic chamber than in 8 × 400 mm tubes (10·48 *vs* 9·54 $\log_{10}$ cfu/g of faeces; $P < 0{\cdot}01$). By contrast, counts measured by the two techniques during nifurzide treatment were not significantly different (Table 4).

Before nifurzide treatment, the dominant anaerobic flora of the mice (including 9·00 $\log_{10}$ cfu/ml) consisted mainly of *Bacteroides spp.*, small and medium size *Eubacterium spp.*, and *Clostridium spp.* with terminal or subterminal spores. During nifurzide treatment anaerobic flora was changed. Only strains of *Bacteroides* spp. could be isolated.

ANTIBIOTIC ACTIVITY IN THE FAECES OF THE HUMAN VOLUNTEERS AND OF THE GNOTOBIOTIC MICE

No antibiotic activity was found in any of 25 faecal samples obtained from the 6 volunteers during nifuroxazide treatment, irrespective of whether the faeces were diluted in saline, acetone, or methanol (Fig. 2). Some antibiotic activity, however, was found in the faeces of the volunteers treated with nifurzide. When the faeces were diluted in acetone this activity was high, reaching 0·46 ± 0·19 and 0·65 ± 0·32 mg/g of faeces for total daily doses of 450 and 900 mg of nifurzide respectively ($P < 0{\cdot}05$).

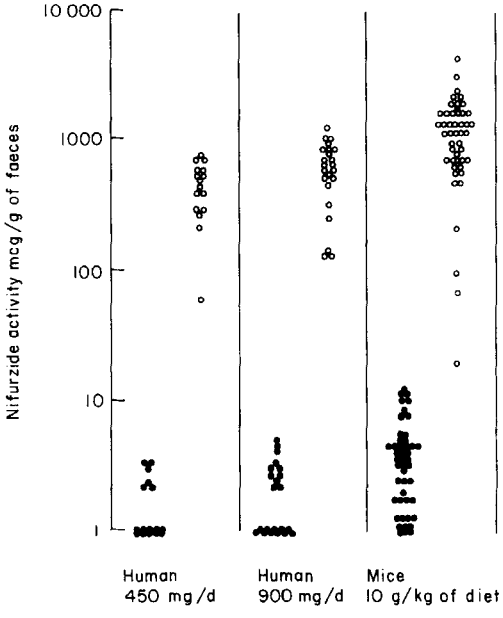

Fig. 2. Faecal activity of nifurzide in human volunteers and in gnotobiotic mice. ●, faeces diluted in saline; ○, faeces diluted in acetone.

When they were diluted in saline, however, the activity was undetectable in 21/38 of the samples (55%) and was low in the remaining 17 samples (3·07 ± 0·81 μg/g). In 46 samples of faeces from mice treated with nifurzide the mean activity was 1·26 mg/g when the faeces were diluted in acetone. As in the human samples, this activity was much reduced when the faeces were diluted in saline. Some activity was noted, however, in 43/46 of the samples tested (93%). The mean activity of the 43 positive mouse samples was slightly higher than that of the 16 positive samples obtained from the human volunteers (4·65 ± 1·15 *vs* 3·07 ± 0·81 μg/g, $P < 0{\cdot}05$).

Table 5. Effect of binding of nifurzide to faecal content and of its solubility on nifurzide activity*

	Solvents			
	Saline	Acetone	Methanol	DMF† acetone
Without faeces	0·9	68	91	112
With faeces	1·2	87	61	87

* Values are expressed in percent of theoretical activity.
† Dimethylformamide.

EFFECT OF THE BINDING OF NIFURZIDE TO INTESTINAL CONTENT AND OF ITS SOLUBILITY ON ANTIBIOTIC ACTIVITY

The use of solvents other than saline was essential to recover the antibiotic activity from solutions of nifurzide. By contrast nifurzide binding to bacterial cells or inert faecal material had no detectable effect (Table 5).

Discussion

As some of the target organisms of nifurzide and nifuroxazide treatments are members of the family Enterobacteriaceae and as the colon of healthy humans is colonized with endogenous nonpathogenic Enterobacteriaceae, we have assessed the effect in human volunteers of the doses usually given in nifurzide and nifuroxazide treatments. No modifications were observed in the faecal concentrations of endogenous Enterobacteriaceae or in their susceptibility to the two drugs. The results of the antibiotic activity assays of the faecal samples suggested that this absence of activity was caused by the poor solubility of the drugs in simple solvents like physiological saline. In the group of volunteers taking nifurzide, high antibiotic activity was found when the faeces were diluted in acetone. When they were diluted in saline, however, such activity was either undetectable or lower than the MICs of nifurzide in the endogenous Enterobacteriaceae (Fig. 2 and Table 2). The results of the reconstitution and solubility experiments confirmed this hypothesis (Table 5). Recovery of antibiotic activity was mainly a function of the solvent used. The presence of faecal material in the samples induced no significant change in this activity. In the group of volunteers who took nifuroxazide no such activity was ever detectable, whether the faeces were diluted in saline, acetone or methanol. Other solvents like chloroform or polyethylene glycol could not be used for sample assay because of their intrinsic antibacterial activity. Significantly higher activity could be detected in the faeces of the mice when they were treated with high doses of nifurzide.

For obvious ethical reasons volunteers could not be used to test the *in vivo* effect of such doses of nitrofurans on intestinal flora and on the microbial antagonisms that prevent intestinal colonization by exogenous micro-organisms. Gnotobiotic mice are convenient for such studies because (a) the mutagenic effect of nitrofurans on eukaryote cells (McCalla 1977) is not a limitation, (b) most bacterial species pathogenic for humans are harmless to mice, thus allowing prolonged periods of observation, and (c) the qualitative and quantitative characteristics of human faecal flora can be reproduced in the intestine of germ-free mice (Raibaud *et al.* 1980; Hazenberg *et al.* 1981). Moreover the effects of several antimicrobial drugs on intestinal bacteria have been successfully tested in this model (Hazenberg *et al.* 1982; Andremont *et al.* 1983; Hazenberg *et al.* 1983).

We used germ-free mice associated with a complex human faecal flora containing no Enterobacteriaceae because two out of the four challenge strains (*E. coli* H10407 and *Sh. flexneri* DKR 115) could not have been differentiated from the endogenous Enterobacteriaceae of a complete human faecal flora. The faecal flora had been previously studied and described. It retained most of its resistance to colonization characteristics (Andremont *et al.* 1983).

Nifurzide was chosen for the experiments with mice because of its good *in vitro* activity (Table 2). When gnotobiotic mice were treated with high doses, the saline-soluble fraction of the faecal antibiotic activity exceeded the *in vitro* MICs of the challenge strains, and biological activity was observed (Fig. 2 and Table 3). The cellular modifications then noted by electron microscopy (Fig. 1) were similar to

those reported in the literature for *in vitro* activity with equivalent concentrations of nifurzide (Delsarte *et al.* 1981).

The effect of nifurzide on gnotobiotic mice associated with the anaerobic flora and later challenged with *V. cholera* 569B and *V. parahaemolyticus* J525C could not be investigated because the challenge strains were rapidly eliminated from the intestine of the animals. It was possible, however, to demonstrate that nifurzide significantly decreased colonization resistance to *E. coli* H10407 and *Sh. flexneri* DKR 115. The inhibitory action of the human flora on these two challenge strains was essentially eliminated by nifurzide. Microbial antagonisms diminished so markedly that the counts of the challenge strains rose to those recorded in the monoassociated mice during the treatment (Table 4).

The normal flora appears to be a major factor affecting the establishment of pathogenic bacteria in the intestinal tract (Hentges 1983). Our results showed that high doses of nifurzide greatly modified the human flora in the intestine of the gnotobiotic mice. Total anaerobic counts performed in the glove box dropped significantly during the treatment (Table 4) and the composition of the dominant anaerobic flora was strikingly simplified. By contrast total counts in 8 × 400 mm tubes did not change, suggesting that only the strains most sensitive to oxygen were affected by nifurzide treatment. In one study two extremely oxygen-sensitive strains of *Clostridium spp.* were shown to be the major constituents of a barrier against *Sh. flexneri* in the digestive tract of gnotobiotic mice (Ducluzeau *et al.* 1977). Such strains are considered to be of primary importance for intestinal colonization by exogenous organisms (Ducluzeau *et al.* 1981).

In conclusion, our results in volunteers suggest that the activity obtained in human faeces with the usually recommended doses of nifurzide and nifuroxazide are too low to induce *in vivo* antibacterial activity on Enterobacteriaceae. Experimental results in gnotobiotic mice suggest that the use of higher doses might theoretically overcome this problem but might also lead to unwarranted modifications of the anaerobic microflora and selection of mutant clones of enteropathogens.

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