Biochemical Effects on Germ-free Mice of Association with Several Strains of Anaerobic Bacteria

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The effects of the following changes throughout the association of germ-free mice with increasing numbers of anaerobic bacteria were studied: (i) elution patterns obtained by gel-filtration chromatography of caecal diffusates; (ii) concentration of β -aspartylglycine in caecal and faecal contents; (iii) polypeptide patterns obtained by sodium dodecyl sulphate–polyacrylamide gel electrophoresis of caecal supernatants; (iv) free amino acid content of caecal supernatants; (v) faecal bile acids, analysed by gas–liquid chromatography; (vi) colonization-resistance. The results indicate that monitoring the normalization (association) process can be accomplished in several ways, but the level of colonization-resistance is most easily measured by high-voltage paper electrophoresis of faecal supernatants to determine the concentration of β -aspartylglycine. During association, the concentration of β -aspartylglycine decreased and became undetectable after association with 40 to 50 different strains of bacteria. There was a good negative correlation between the level of colonization-resistance and the concentration of β -aspartylglycine.

INTRODUCTION

Van der Waaij *et al.* (1971) and van der Waaij & Berghuis (1974) found that germ-free mice contaminated with the intestinal flora of antibiotic-treated mice showed a high resistance against oral contamination with *Escherichia coli*. The intestinal tract of these mice and their offspring contained an exclusively anaerobic microflora of which the precise composition is unknown. These mice were called colonization-resistance factor (CRF) mice.

This paper describes changes that occur after association of germ-free (GF) mice with an increasing number of different strains of anaerobic bacteria isolated from the CRF microflora. These changes include the concentration of the dipeptide β -aspartylglycine, which is unique to the caecal contents of germ-free and antibiotic-treated mice (Welling & Groen, 1978), the elution pattern obtained after fractionation of caecal diffusates on Sephadex G-25 (Welling & Groen, 1978), the free amino acid content of caecal supernatants in sodium dodecyl sulphate (SDS)–polyacrylamide gels and the ratio of secondary to primary faecal bile acids. The relationship of these changes to changes in colonization-resistance was investigated.

METHODS

Animals. Adult Swiss/Cpb:SE mice with a CRF microflora (CRF mice) were used to isolate anaerobic caecal bacteria. Swiss/Cpb:SE mice were used to study normalization comparing germ-free mice, germ-free mice associated with an increasing number of bacterial strains isolated from the CRF microflora (GF+14, GF+17, GF+23, GF+41, GF+46, GF+55, i.e. germ-free mice associated with 14 bacterial strains etc.) and CRF mice. These mice were kept under germ-free isolation conditions in plastic isolators, fed with autoclaved SRM food (Hope Farms, Woerden, The Netherlands) and supplied with autoclaved drinking water. Swiss/Cpb:SE mice with a specific pathogen-free (SPF) microflora (SPF mice) served as controls. These mice were fed with RMH food (Hope Farms, Woerden, The Netherlands), supplied with normal tap water and housed under clean conventional conditions.

Caecal and faecal supernatants. Caecal contents of mice were homogenized and dry weight was determined after drying in a vacuum oven at 90 °C. The caecal contents were diluted to 15 % (w/v) dry wt with water and centrifuged at 27000 g for 30 min in a Sorvall RC-5 centrifuge. Faecal supernatants were prepared by homogenizing faeces (67 mg ml⁻¹) in water. After centrifugation at about 2000 g for 30 min, the supernatants were lyophilized and stored at -25 °C.

Gel filtration of caecal diffusates. Caecal diffusates were prepared by dialysing 0.5 ml of caecal supernatants at 0 to 2 °C in Spectrapor-3 membrane tubing (Spectrum Medical Industries, Los Angeles, Calif., U.S.A.; molecular weight cut-off 3500) against 50 ml demineralized water. Diffusates were fractionated on a column (0.9 × 160 cm) of Sephadex G-25 (fine grade) and eluted with 0.1 M-acetic acid at a flow rate of 10 ml h⁻¹; 2.3 ml fractions were collected and the absorbance at 280 nm was determined.

Semi-quantitative determination of β -aspartylglycine. Caecal supernatants (10 μ l) or faecal supernatants (50 μ l) were subjected to high-voltage paper electrophoresis on Whatman 3MM chromatography paper at pH 3.5 (pyridine/acetic acid/water; 1:10:89, by vol.) for 60 to 90 min at 3 kV. In order to determine the relative concentration of β -aspartylglycine, 10, 5, 2.5 and 1 μ l of caecal supernatant from germ-free mice was also subjected to electrophoresis. β -Aspartylglycine from Serva (Heidelberg, Germany) was used as a reference together with 20 common amino acids. Commercial β -aspartylglycine (dry powder) is in the cyclized α,β -form giving a neutral spot on electrophoresis at pH 3.5 (Welling & Groen, 1978). In aqueous solution it slowly converts to the β -form (Naughton *et al.*, 1960). After electrophoresis the paper was dried at 70 to 80 °C for 10 to 15 min and sprayed with 0.2 % (w/v) ninhydrin in ethanol. Further heating at 70 to 80 °C resulted in purple spots for most of the amino compounds, except for β -aspartylglycine which was greyish. Additional heating at 150 °C for 10 min gave a clear blue spot for β -aspartylglycine.

Relative caecal weight. Caeca were removed and weighed with their contents. Weights were expressed as a percentage of the body weight.

Gastro-intestinal colonization-resistance. Overnight cultures of a mouse-derived streptomycin-resistant strain of *Escherichia coli* (O₈₁) grown in brain-heart infusion broth (Difco) were serially diluted 10-fold. Germ-free mice, germ-free mice associated with the CRF microflora, CRF mice and SPF mice received 10⁸ *E. coli* cells orally. After 3, 5 and 13 d, the concentration of the streptomycin-resistant *E. coli* in the faeces was determined as described earlier (Koopman *et al.*, 1977). Colonization-resistance is expressed as log₁₀(no. of *E. coli* g⁻¹).

Microbiology. To isolate anaerobic caecal bacteria, mice were killed by cervical dislocation and introduced into an anaerobic glove box. The caecum with contents was then removed under aseptic conditions, transferred into 4.5 ml sterile saline and homogenized with an Ultra-Turrax homogenizer (type TP 10N, Janke und Kunkel K.G., West Germany). From a 10⁻⁷ dilution, 100 μ l was spread on to the surface of an agar medium (Wensinck & Ruseler-van Embden, 1971) to which 7 % (v/v) sheep blood was added. The incubation period was 1 week. The anaerobic bacterial species used for association of germ-free mice were characterized with a commercially available rapid test method (API ZYM; API, La Balme-les-Grottes, Montallieu-Vercieu, France) for the assay of the activities of 19 hydrolytic enzymes. The bacteria were given orally and rectally and allowed to colonize for at least 2 weeks before testing the mice. Mice were associated in a stepwise manner with an increasing number of different strains of anaerobic bacteria (J. P. Koopman & H. M. Kennis, unpublished results).

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli (1970) in slab gels (16 × 25 cm) with a linear polyacrylamide gradient from 7 to 20 % (w/v). Markers (cytochrome *c*, lysozyme, chymotrypsinogen, ovalbumin, H chain of IgG, bovine serum albumin and β -galactosidase) were used ranging from 12000 to 116 000 in molecular weight. Electrophoresis was performed at room temperature for 22 to 24 h at a constant current of 11 to 12 mA.

Amino acid analysis. Amino acid compositions were determined with a Kontron Liquimat III amino acid analyser (Kontron, München, West Germany) using norleucine as an internal standard.

Gas-liquid chromatographic (g.l.c.) analysis of faecal bile acids. To determine faecal bile acids, 0.3 g of

pooled faeces was suspended in distilled water, adjusted to pH 1 with HCl, and extracted three times with about 20 ml diethyl ether. The extracts were evaporated to dryness and resuspended in 5 ml 0.1 M-acetate buffer, pH 5.7, by ultrasonic vibration. Deconjugation was achieved by overnight incubation at 37 °C with 0.1 ml cholyglycine hydrolase (Schwartz-Mann, Orangeburg, N.Y., U.S.A.), after which bile acids were extracted with HCl and diethyl ether as described above. Trifluoroacetic acid (TFA) derivatives of methylated bile acids were prepared for g.l.c. analysis. Separations were performed on a F & M 401 gas chromatograph (Hewlett-Packard, Avondale, Penn., U.S.A.) using a column packed with 3% (w/w) OV210 on 100 to 120 mesh Gas-Chrom Q (column temperature, 235 °C; inlet and detector temperature, 250 °C; carrier gas, N₂). TFA derivatives were identified by comparing their relative retention times with those of reference compounds. The TFA derivative of methylated 7-ketodeoxycholic acid (0.4 µmol) was added to each sample as an internal standard.

RESULTS AND DISCUSSION

After association with at least 40 strains of bacteria, the relative caecal weight of germ-free mice was reduced almost to that of CRF mice (Table 1). After association with 40 to 50 strains of bacteria, β -aspartylglycine was almost undetectable in faecal and caecal supernatants. No β -aspartylglycine was found in faecal and caecal supernatants from CRF and SPF mice.

The level of gastro-intestinal colonization-resistance to *Escherichia coli* was determined (Table 1); the relationship between colonization-resistance and the concentration of β -aspartylglycine was nearly linear. Unexpectedly, intermediate values of colonization-resistance were found in CRF mice. This is in contrast to the results reported by van der Waaij *et al.* (1971).

Comparison of free amino acid concentrations in caecal supernatants from germ-free mice associated with 14 and 23 strains of bacteria, CRF mice, germ-free mice and SPF mice (Table 2) showed that presence of bacteria in the intestinal tract was associated with higher concentrations of most amino acids except glycine. In SPF mice, however, the concentration of glycine was about 50% of that found in the other types of mice. Strikingly high concentrations of free histidine and, to a lesser extent, of arginine were found in caecal supernatants from the mice associated with different strains of bacteria (Table 2).

Figure 1 shows the elution pattern obtained after gel filtration of caecal diffusates from CRF mice and germ-free mice associated with 23 strains of bacteria; it also shows part of the elution pattern typical of diffusates from germ-free and SPF mice. The peaks eluting in

Table 1. *Comparison of relative caecal weight, relative concentration of β -aspartylglycine and the level of colonization-resistance after stepwise association of germ-free mice with an increasing number of strains of anaerobic bacteria*

Values are averages $\pm$ standard deviation.

Type of mice and microflora*	No. of mice	Relative caecal weight (% of body wt)	Relative β -aspartylglycine concn (arbitrary units)	Colonization-resistance [$\log_{10}$ (concentration of resistant <i>E. coli</i>)]
GF	59	5.5 $\pm$ 0.8	15	7.6 $\pm$ 0.9
GF+17	12	3.7 $\pm$ 0.8	10	6.2 $\pm$ 0.7
GF+41	19	3.0 $\pm$ 0.9	6	4.1 $\pm$ 1.2
GF+46	38	2.5 $\pm$ 0.5	1	2.3 $\pm$ 0.7
GF+55	28	2.5 $\pm$ 0.4	1	2.4 $\pm$ 0.6
CRF	46	2.1 $\pm$ 0.4	0	5.1 $\pm$ 1.4
SPF	16	1.3 $\pm$ 0.4	0	ND

ND, Not determined.

* GF, germ-free mice; GF+17, germ-free mice associated with 17 strains of anaerobic bacteria etc.; CRF, colonization-resistance factor mice; SPF, specific pathogen-free mice.

Table 2. *Free amino acid composition of caecal supernatants*

Caecal contents were diluted to 15% (w/v) dry wt and centrifuged before determination of amino acid composition as described in Methods. The concentration of proline was not determined, since some unknown compound was eluted from the column of the amino acid analyser at the same position as proline.

Amino acid	Amino acid content ($\mu\text{mol ml}^{-1}$)				
	Type of mice and microflora*				
	GF	GF+14	GF+23	CRF	SPF
Asp	4.7	5.6	8.1	6.8	6.7
Thr	6.5	5.3	8.2	6.8	5.4
Ser	11.8	6.8	9.4	7.7	8.6
Glu	8.9	12.3	21.7	18.3	12.2
Gly	12.8	11.3	13.1	10.5	6.4
Ala	10.7	15.5	21.8	18.5	10.7
Val	6.5	7.0	9.8	9.0	7.0
Met	1.4	2.0	2.8	2.9	2.7
Ile	4.0	4.6	6.6	6.6	5.6
Leu	6.6	8.3	12.5	10.6	8.9
Tyr	2.6	2.7	4.5	4.0	2.7
Phe	3.1	3.3	5.4	4.2	4.4
Lys	7.7	10.4	14.8	11.7	8.8
His	2.7	9.2	9.2	0.9	1.5
Arg	4.2	4.6	6.8	1.1	3.3

* Abbreviations as in Table 1.

Fig. 1. Gel filtration of diffusates of caecal supernatants from (a) CRF mice and (b) germ-free mice associated with 23 different strains of anaerobic bacteria on a Sephadex G-25 (fine) column (0.9×160 cm). Elution was carried out with 0.1 M-acetic acid at a flow rate of 10 ml h^{-1} , 2.3 ml fractions were collected and the A_{280} was determined (●). For comparison, part of the elution patterns obtained with diffusates from GF (germ-free) and SPF (specific pathogen-free) mice are also shown (—).

Table 3. *Faecal excretion of bile acids by germ-free mice associated with anaerobic bacteria*

Bile acid contents of ether extracts were determined by g.l.c. as described in Methods.

Bile acid	Bile acid content [$\mu\text{mol (g wet faeces)}^{-1}$]						
	Type of mice and microflora*						SPF
	GF	GF+17	GF+41	GF+46	GF+55	CRF	
Primary bile acids							
Cholic acid	3.84	4.26	5.38	5.11	3.27	2.39	0.34
β -Muricholic acid	0.26	0.99	0.45	1.26	0.91	0.92	0.01
Secondary bile acids							
Hyodeoxycholic acid	0	0.36	0.65	0.52	0.41	0.57	0.04
7-Ketolithocholic acid	0	0	2.54	3.76	2.28	3.88	0.39
Deoxycholic acid	0	0	0	0	0	1.68	0.52
Lithocholic acid	0	0	0	0	0	0	0.26
Unidentified	0	0	0	0	0	0	0.42
Total	4.10	5.61	9.02	10.65	6.87	9.44	1.98

* Abbreviations as in Table 1.

Table 4. *Microbial activity of anaerobes in germ-free mice as indicated by the formation of secondary bile acids*

Type of mice and microflora*	No. of different secondary bile acids	Ratio of amounts of secondary to primary bile acids
GF	0	0
GF+17	1	0.07
GF+41	2	0.55
GF+46	2	0.67
GF+55	2	0.64
CRF	3	1.84
SPF	6	4.82

* Abbreviations as in Table 1.

fractions 66 to 70 are of especial interest. It was found earlier (Welling & Groen, 1978) that the peak at fraction 70 is characteristic of caecal diffusates from SPF mice, while the peak at fraction 66 is typical of caecal diffusates from germ-free mice. Caecal diffusates from CRF mice contained material eluting in the same position as diffusates from SPF mice, while diffusates from germ-free mice associated with 23 strains contained material eluting in fractions 68 and 69. The latter peak was not symmetrical and seemed to contain several components. This again shows that the germ-free mice associated with 23 strains were at an intermediate stage in the process of normalization. Similar results were observed with diffusates of germ-free mice associated with 14 strains of bacteria (not shown).

SDS-polyacrylamide gel electrophoresis showed that caecal supernatants from germ-free mice had polypeptide patterns quite different from those of other types of mice used (see Fig. 2). After association with 9 or 10 different strains of bacteria, the polypeptide pattern somewhat resembled that of CRF and SPF mice (not shown). However, some differences from controls (SPF mice) remained even after association with more strains of bacteria, as can be seen by comparing the high molecular weight regions ($> 116\,000$) in Fig. 2.

The faeces of germ-free mice contained only the primary bile acids muricholic acid and cholic acid (Table 3). With increasing complexity of the intestinal flora an increasing number of secondary bile acids appeared. Table 4 summarizes two sets of values that may indicate the degree of normalization of the intestinal flora. The number of secondary bile acids in the faeces is a measure of the metabolic versatility of the intestinal microflora.

Fig. 2. SDS-polyacrylamide gel electrophoresis of caecal supernatants from (1) SPF mice, (2) germ-free mice, (3) CRF mice, (4) germ-free mice associated with 23 different strains of anaerobic bacteria and (5) germ-free mice associated with 14 different strains of anaerobic bacteria. In the sixth slot, markers were applied with molecular weights ranging from 12 000 to 116 000. Generally 10 μ l of a 15% (w/v) dry wt caecal supernatant was subjected to electrophoresis as described in Methods.

Bacterial hydroxycholesterol dehydrogenases active on substituents at the 3, 7 and 12 position have been described (Aries & Hill, 1970; Macdonald *et al.*, 1976). Bacterial dehydrogenation of the bile acid nucleus and aromatization of the steroid rings were described by Goddard & Hill (1972) and Goddard *et al.* (1975). On the other hand, the ratio of secondary to primary bile acids in the faeces measures the metabolic potential of the gut flora. The increase in this ratio (Table 4) suggests an increasing microbial degradation of the bile acids with increasing complexity of the intestinal flora. Even after association with 55 strains of bacteria the ratio of secondary to primary bile acids did not return to the level of CRF mice. This indicates that the normalization as a result of association of germ-free mice with an increasing number of strains of bacteria is a very complex process. Some values returned to normal levels (β -aspartylglycine) earlier than others (ratio of secondary to primary bile acids).

The present study shows that determination of the relative concentration of β -aspartylglycine in the faeces and the ratio of secondary to primary bile acids by g.l.c. analysis are suitable methods for assessing the state of normalization and have the advantage that they can be applied to individual mice throughout the whole process of normalization, since excision of the caecum is not necessary. Colonization-resistance correlates well with β -aspartylglycine content, so that monitoring normalization of colonization-resistance is most easily accomplished by high-voltage paper electrophoresis of faecal supernatants to determine the relative concentration of β -aspartylglycine. Preliminary experiments indicate that similar bacteriological situations exist in patients treated with particular antibiotics. Totally decontaminated patients show relatively high concentrations of β -aspartylglycine in the faeces in contrast to healthy controls (Welling, 1979).

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